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**THE EFFICIENCY OF NITROGEN UTILISATION
IN GROWING CHICKS**

CLARE LOUISE RICHARDSON

**A thesis submitted in partial fulfilment of the
requirements of the Open University
for the degree of Master of Philosophy**

January 1998

Harper Adams Agricultural College

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Abstract

High levels of nitrogen excretion result in economic loss in farm animal production. Recent theories have proposed that there is a poor agreement between amino acid oxidation and the rate of protein synthesis. The overall objectives of three separate experiments were to quantify the growth, efficiency of feed and nitrogen utilisation and rate of lysine oxidation in growing chickens. The first experiment compared the growth and efficiency of nitrogen utilisation of birds given eight different dietary crude protein concentrations (130 - 300g/kg). Increasing dietary protein had no effect on weight gains ($p>0.05$) but an inverse relationship ($p<0.001$) with the efficiency of nitrogen utilisation. However, the results indicated that diets with increasing protein concentrations had poor protein digestibility. A second experiment compared eight dietary crude protein concentrations (129-305g/kg) each at six lysine concentrations (40-65g/kg of protein). There was a curvilinear increase ($p<0.001$) in growth rates with increasing dietary protein with maximum growth rates occurring at 280g/kg. There was also a quadratic effect ($p<0.01$) of lysine concentration on weight gain with the maximum growth occurring at 55g/kg. The optimum crude protein

concentration for maximum growth was indicated at 275 g/kg diet and there was no effect of protein concentration ($p>0.05$) on the efficiency of nitrogen utilisation. The third experiment examined the response of growing chickens to a diet that varied only in lysine concentration (30-100g/kg of protein). The optimum lysine concentration for maximum growth was indicated to be 64g/kg CP and the optimum for maximum efficiency of nitrogen utilisation was also indicated at 64 g/kg CP. Therefore there was no evidence that maximum weight gain and maximum nitrogen retention occurred at different protein and lysine concentrations. Lysine oxidation increased with an increase in dietary lysine and there was evidence of a curvilinear response. A rapid increase in lysine oxidation was indicated at 55g/kg CP. This data therefore gives some support to the theory of increased oxidation of amino acids prior to the point of maximum protein retention in poultry.

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CHAPTER 1

GENERAL INTRODUCTION

1.0 General Introduction

High levels of nitrogen excretion result in economic loss in farm animal production. The animal production industry has always been concerned with improving protein digestibility and improving the efficiency of protein metabolism within the body, but recently there has been a greater awareness of the environmental concerns (Tamminga and Verstegen, 1992). Poultry are fed relatively high levels of dietary protein compared to other farm animals and approximately 60-70 % of the nitrogen consumed is excreted. This is clearly a wasteful and significant loss to the environment, which results in poultry operations being a potential source of pollution (Summers et al., 1993).

Broiler production in the UK in 1995 resulted in 73, 000 tonnes of nitrogen being released into the environment. Gaseous nitrogen losses in the form of ammonia, regularly reaches high enough concentrations in Europe's farm buildings to harm both farm workers and animals and particularly in intensive poultry units (New Scientist, 1997). The processes of nitrification and denitrification also results in the production of nitrates and oxides which not only contribute to the depletion of the ozone layer but also pollute the water

supply.

There is clearly a problem which requires research into the reduction and more efficient utilisation of nitrogen. Research is currently being conducted into the reduction of nitrogen excretion, for example the use of low protein diets substituted with synthetic amino acids. Often the objectives of this research and the objectives of the poultry industry are to maintain maximum levels of growth and feed utilisation. However, it may be that the diets and feeding programmes that give maximum efficiency of nitrogen utilisation may not be able to achieve maximum growth and feed utilisation. Amino acids which are the monomers of protein are either incorporated into protein or oxidised and it is the partition between these two routes that determines protein deposition or nitrogen excretion. Some theories suggest a poor agreement between amino acid oxidation and protein synthesis such as the anabolic drive theory proposed by Millward and Rivers (1988). The consequence of the hypothesis proposed by these authors is that, as the point of maximum protein deposition is reached, amino acid oxidation has risen significantly above basal rates. Hawkey (1994) examined these effects with *in vitro* studies using rats and also found evidence of raised amino acid oxidation rates when protein deposition reached maximum levels. If this

relationship occurs in poultry, then it could indicate that future dietary and management strategies should be adopted in commercial production systems. These strategies will not attempt to maximize growth and protein deposition rates, but accept lower rates that give less amino acid oxidation and thus maximize the efficiency of nitrogen utilisation in the birds.

There is clearly a need to examine whether diets that give maximum rates of growth and feed utilisation efficiency also give the maximum efficiency of nitrogen utilisation. Therefore the objectives of this thesis were to quantify the growth and efficiency of feed utilisation and efficiency of nitrogen utilisation of growing broiler chickens. The chickens were fed diets that varied in crude protein concentration and varied in amino acid quality within that protein. Lysine was selected as the amino acid to examine. The second objective was to quantify the rate of lysine oxidation in diets that varied in lysine concentration.

CHAPTER 2

ENVIRONMENTAL CONSEQUENCES OF NITROGEN IN ANIMAL PRODUCTION SYSTEMS

2.0 Environmental Consequences of Nitrogen Derived from Animal Production Systems.

2.1 Nitrogen pollution from livestock production systems

Most non-ruminant animal production in developed countries is carried out in intensive production systems. Intensive animal production systems have a number of negative effects on the environment and this is potentially important in countries or areas with high concentrations of these production units, for example the Netherlands Germany, Britain and Belgium.

Nitrogen is introduced to the animal in the form of feed protein and excreted in the form of undigested dietary protein and endogenous nitrogen that is a combination of desquamated gut wall cells and microbial nitrogen in the faeces and uric acid or urea in the urine. Only a small part of the protein fed to farm animals is retained in the body tissues or excreted in milk and eggs (Figure 2.1). Poultry can be fed up to 28% crude protein diets which are relatively high compared to other farm animals, therefore these operations can be a potential source of environmental pollution (Summers *et al.*, 1993). Approximately 60-70% of nitrogen consumed by poultry is excreted and this represents a significant loss of nitrogen to the environment (Figure 2.2).

Figure 2.1: Nitrogen utilisation in different species of farm animals.

Animal	Nitrogen content as a proportion of dietary nitrogen intake(%)			
	Body	Milk + Eggs	Faeces	Urine
Lactating cow, grazing	2	17	25	56
Lactating cow, stall fed	2	25	34	39
Beef cattle	22	-	30	48
Veal calf	54	-	5	41
Lactating sow	5	20	20	55
Piglet	40	-	10	50
Growing pig	32	-	15	53
Laying hen	2	30	12	56
Broiler	42	-	10	48

Source: Adapted from Tamminga (1992a)

In its lifetime, each broiler consumes approximately 4600g of feed, therefore 160g of nitrogen. Only 60g of this nitrogen will be retained, so the remainder (100g/bird) is excreted. The U.K broiler production sites are generally large with 57% of the national flock kept in units consisting of 100,000 birds or more. Additionally, U.K broiler production is concentrated in areas close to processing plants. Processing plants tend to be clustered together in areas such as East Anglia. The severity of environmental pollution from poultry units can be acute in certain areas of the UK. In 1995 there were approximately 730 million broilers produced in the U.K, so that the industry resulted in approximately 73,000 tonnes of nitrogen being released into the environment that year.

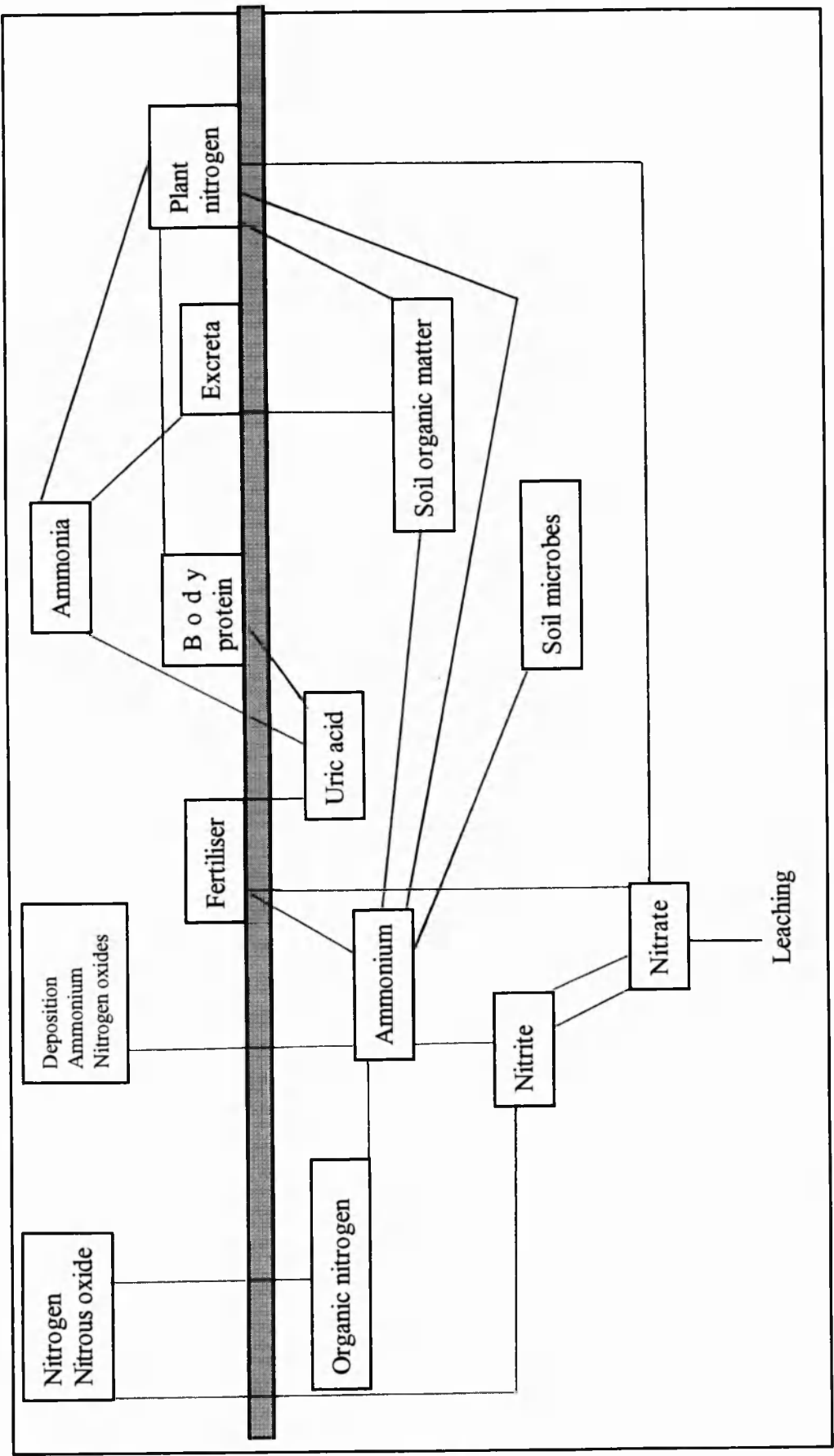
This problem is also of major concern in other E.U countries. In the Netherlands, Heij and Schneider (1991) estimated that ammonia of which more than 90% was produced by agricultural activities, was responsible for about 45% of the acid deposition in the Netherlands. Nitrate concentrations in underground waters indicate that from about 40% of the land used for agriculture, the nitrate leaching is higher than the E.C. limit for drinking water of 50 mg of nitrate per litre (RIVM, 1991).

It is the ammonia production and water losses that are the two ways in which nitrogen in excreta is a vast problem. Between 60 and 75% of nitrogen in excreted manure is converted to ammonia (Klarenbeek and Bruins, 1988) during storage and spreading. Van de Meer (1991) estimated that in 1986, about 225 million kg of ammonia was lost during storage, spreading and in pasture, which equals about 30-40% of the total nitrogen excretion from animal production in the Netherlands .

The recommended safety level for airborne ammonia is 20ppm for animals and 25ppm for people which is frequently exceeded. The highest single concentration reached 73ppm in a Dutch battery farm and the average airborne ammonia concentration in four British broiler houses was 27ppm (Coghlan, 1997)

Soil and ground surface water can be polluted by compounds from slurry, farm yard manure, dirty water and silage effluent. When water is polluted by such materials, the microbial degradation of organic matter can rapidly consume a large part of the oxygen in the water. Due to this high biological oxygen demand their presence in the water causes a negative impact on aquatic life (Webb and Archer, 1994). About 75% of the nitrogen input is lost

Figure 2.2 The nitrogen cycle in poultry production (adapted from Groot Koerkamp, 1994)



due to emission, denitrification, leakage and accumulation with estimates of the nitrogen surplus ranging from 320-400 kg/ha (RIVM, 1991).

2.1.1 Gaseous Nitrogen losses

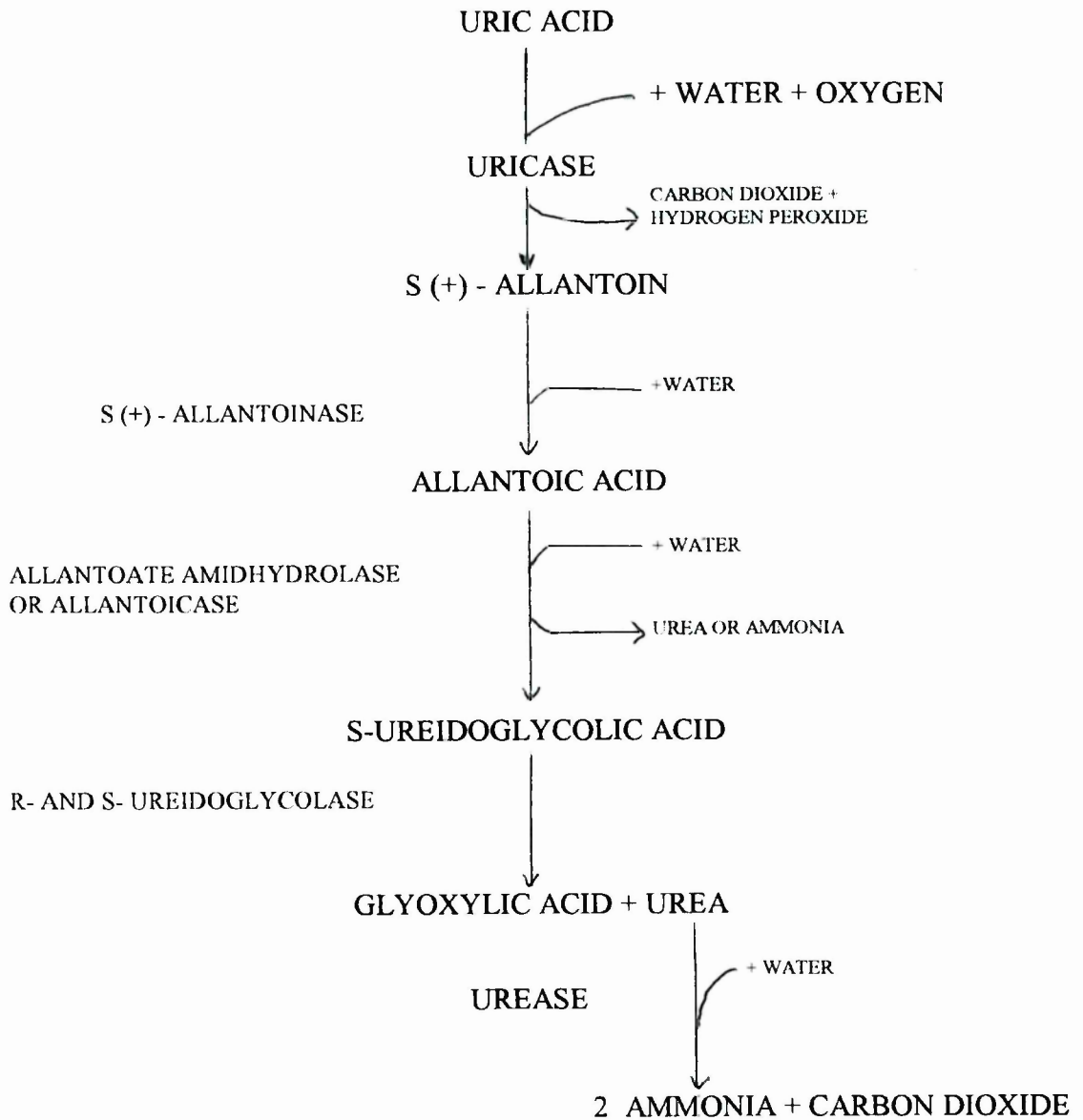
Gaseous nitrogen losses arise predominantly from aerobic bacterial fermentation of poultry excreta. Excreta is a mixture of faecal material and urine. Poultry excrete nitrogen in their urine in the form of uric acid.

The uric acid is rapidly converted to ammonia (Figure: 2.3) by the urease activity of the faecal microbes which escapes easily into the air by volatilisation. The degradation of uric acid to ammonia lies between 8-40% of the uric acid per day, for dry and liquid poultry manure respectively (Groot Koerkamp, 1994). This indicates the dependence of the ammonia release rate on litter moisture content with water being necessary for the growth of the microbes involved in the degradation.

Part of the ammonia is converted to gaseous nitrogen (N_2) by the processes of nitrification and denitrification (Section: 2.1.2). This results in the formation of intermediate oxides NO_2 , NO and N_2O , a proportion of which escapes to the atmosphere. It is these nitrogen oxides together with the

ammonia which contribute to the acid deposition and impairment of the ozone layer.

Figure 2.3 Aerobic decomposition of uric acid.



(Groot Koerkamp, 1994)

2.1.2 Aqueous nitrogen losses

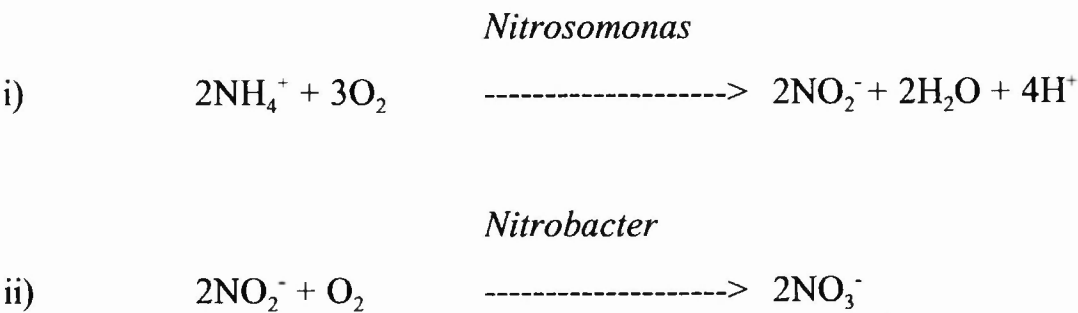
After manure has been applied to the soil, water soluble nitrate is formed from organic nitrogen and ammonia by mineralisation and nitrification. Ammonia is the starting point for the nitrification process which involves the oxidation of the ammonia to nitrite by the action of the aerobic bacteria *Nitrosomonas* and the conversion of nitrite to nitrate by the action of *Nitrobacter* (Figure 2.4). The nitrate produced can be used by plants or lost by denitrification and leaching. Denitrification refers to the reduction of nitrate to nitrite, nitrous oxide, nitric oxide, ammonia and gaseous nitrogen (Figure 2.5).

The nitrate is reduced to nitrite by the action of nitrate reductase (A). The action of nitrite reductase (B) results in the formation of an unstable intermediate, hyponitrite which is then reduced by hyponitrite reductase (C) to form nitrous oxide. At low temperatures, nitrogen gas is formed. The effect of temperature on the formation of the end products is illustrated by the action of *Denitrobacillus* which produces larger quantities of nitrous oxide at higher temperatures. The reduction of the hyponitrite to ammonia requires microaerophilic conditions and uses hydrazine as an intermediate. The microorganisms involved in this reaction include, *Bacillus subtilis* and some

Azotobacter species.

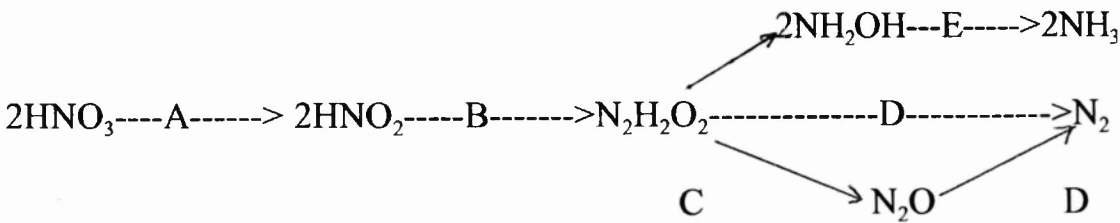
Some of these products are reincorporated into the soil environment (NO_2 , NH_3). Others are released in the gaseous form (N_2 , NO , N_2O). The bacteria involved in nitrate reduction are anaerobic heterotrophs of the species of the genera *Pseudomonas*, *Micrococcus* and *Bacillus*, which are of great abundance in the soil and perform aerobic activities until the oxygen becomes scarce. Therefore the potential for denitrification is enormous (Higgins and Burns, 1975).

Figure 2.4: The Nitrification Process.



(Higgins and Burns, 1975)

Figure 2.5: The Denitrification Process



(Higgins and Burns, 1975)

2.2 Conclusions

- i) The nitrogen content of poultry diets is high relative to other farm animal feeds.
- ii) Efficiency of nitrogen retention in poultry production is very low giving high nitrogen losses to the environment causing concern for the health and welfare of both man and animal.
- iii) High levels of nitrogen excreta from intensive poultry units results in large losses of gaseous ammonia and aqueous nitrates.
- iv) There is a need to examine methods that can improve the utilisation of dietary nitrogen inputs in poultry units.

CHAPTER 3

NITROGEN UTILISATION IN POULTRY

3.0 Nitrogen Utilisation in Poultry

3.1 Nitrogen Supply (Input)

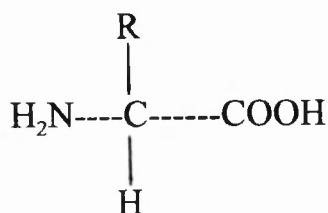
Nitrogen is supplied to the animal in the form of protein and non protein nitrogen compounds, with protein requirements ranging from 230 g/kg diet for broilers 0 to 3 weeks of age and 180g/kg diet for broilers 6 to 8 weeks of age (NRC, 1994). The most appropriate dietary crude protein content of feed for poultry is constantly under investigation and may decrease in the future, due to the improved availability of relatively inexpensive sources of individual amino acids to use in proprietary diets.

Interest is growing in the study of minimum dietary protein levels to optimise output rather than using input levels to maximise output (Summers *et al.*, 1993) and efforts to reduce dietary protein by proper amino acid supplementation have been the subject of numerous investigations (Keshavarz and Jackson, 1991). A number of factors may be involved in the determination of an optimal dietary protein concentration and one of these is the cost of the diet. If it becomes less expensive due to more amino acid supplementation then lower protein diets will become more economically viable.

3.1.1 Amino acids

Although these are technically non protein nitrogen compounds, amino acids are the building blocks of protein. All proteins are polymers and the monomers that combine to make them are amino acids. There are twenty different amino acids that have been incorporated into proteins, each consisting of a carbon skeleton, an amino group, a carboxyl group, a hydrogen atom and a side chain (R). It is this side chain which distinguishes the amino acids from each other.

Figure 3.1 :Amino acid structure.



Amino acids serve a variety of functions in the body in addition to providing the monomers from which proteins are synthesised. Examples of their secondary roles include the provision of energy , the synthesis of hormones and the synthesis of certain constituents of bile. In intermediary metabolism amino acids are used for protein synthesis as a precursor of non protein

substances or as an energy source (Fuller, 1991).

A minimum amount of amino acids (10-40%) are lost (oxidised) due to maintenance requirements and some of the total amino acid input is unavoidably lost in an inefficient utilisation of amino acids for protein synthesis in the body, milk and eggs known as inevitable amino acid catabolism (Tamminga and Verstegen, 1991).

3.1.1.1 Essential and non essential amino acids.

When discussing amino acid utilisation it is necessary to define essential and non essential amino acids. The definition of an essential amino acid is one which cannot be synthesised by the body therefore must be included in the diet. An amino acid is essential if under normal conditions dietary supply of the amino acid is necessary for normal growth. The limiting amino acid is the amino acid of which extra supply enables extra growth. In poultry diets the amino acids most likely to be limiting in their supply are lysine and methionine.

3.1.2 Non protein nitrogen compounds

Such nitrogen compounds include nitrogenous lipids, amines, amides,

purines, pyrimidines nitrates and alkaloids. These are present in low levels so are not considered important.

3.1.3 Digestion of Nitrogen Compounds

The purpose of this section is to explain the input of nitrogen into the body via the process of digestion. After ingestion via the mouth and crop the protein is transported to the proventriculus where the process of dietary protein digestion is initiated by the action of the hydrolytic enzyme pepsin.

Pepsin is secreted in the proventriculus as the inactive precursor pepsinogen and is converted to the active form in the presence of hydrochloric acid, which provides the optimum pH for the pepsin activity to occur. As the food moves quickly through the proventriculus, it is held for a long time in the gizzard so most of the protein hydrolysis by pepsin occurs in this part of the digestive tract. The acidic conditions of the proventriculus and gizzard lead to the breakdown of the 3-dimensional structure of the protein to expose the bonds which are peptide sensitive.

The polypeptides resulting from the action of pepsin are further hydrolysed on entering the small intestine by endopeptidases- trypsin and chymotrypsin

and exopeptidases. These enzymes are secreted into the small intestine by the pancreas as inactive precursors and it is the protease and enterokinase excreted by the intestinal wall which initiates activation by converting trypsinogens to trypsins. The trypsins formed, then initiate the activation of the other inactive precursors. Therefore trypsins are central to the development of full proteolytic activity.

In addition to the enzymes in the animal, there are enzyme activities associated with the bacteria of the gut. In the chick, bacterial activity occurs throughout the tract, but is thought to be concentrated in the caeca (McNab, 1973). Attempts to quantitate this contribution have indicated that it is not quantitatively substantial (McNab, 1973; Salter et al., 1974) however although Ratcliffe (1991) stresses the importance of gut microorganisms in the digestion of protein, the prevailing opinion is to use caeectomised birds in the study of amino acid digestibility (McNab, 1994).

Following their respective enzymatic reactions, substances released during digestion are in the form of either free amino acids or oligopeptides. These substances then pass through the intestinal wall where they become modified or are used for tissue synthesis; the free amino acids move against a

concentration gradient via the process of active transport and the oligopeptides enter the mucosal cells where they are hydrolysed before being transported to the blood in the form of amino acids. The products are then transported to the liver via the hepatic portal system.

3.2 Absorbed Nitrogen Losses (Output)

Feed nitrogen not deposited in the animal is lost as undigested, endogenously excreted, inevitable losses from stored body protein (body protein turnover), or the products of deamination due to the inability of the bird to deposit the supplied protein.

3.2.1 Losses due to incomplete digestion.

Digestibility of the feedstuff may be defined as the proportion of the feed which is not excreted in the faeces and is therefore assumed to be absorbed by the animal (McDonald, Edwards and Greenhalgh, 1994). As feed is one of the major costs of poultry production, the efficiency of feed conversion for the production of meat or eggs is of great importance. This depends upon the efficiency with which the feed is digested and absorbed.

True digestion increases to over 80% before the end of the ileum (Wunsche *et al.*, 1987; Huisman, 1990). However, low digestion may occur due to a number of reasons.

3.2.1.1 Factors affecting digestibility of protein

There are a number of major factors affecting the digestibility of the dietary

components of a feedstuff. These are, the presence of proteolytic enzymes in the gastrointestinal tract, the enzyme's ability to access the substrate for reaction to take place, the ability of the gastrointestinal tract to absorb the products of hydrolysis, the passage time of the ingesta and the presence in the feedstuff of antinutritive factors (ANF's) which may change the structure of the dietary components or interfere with the action of the digestive enzymes.

There is a need for the presence in the digestive tract of enzymes capable of hydrolysing the dietary proteins. In young birds a lack of adaptation to the diet by the digestive organs may result in insufficient proteolytic enzymes (Corring *et al.*, 1989).

There is also the requirement for the ability of the digestive enzymes to gain access to the proteins. The inhibition of proteolytic enzymes (Birk, 1989) is a serious problem that involves trypsin inhibitors. Trypsins are central to the development of full proteolytic activity by initiating proteolysis and therefore the consequence of trypsin inhibition results in a decrease in the digestibility of the amino acids contained in the dietary protein. Soyabeans contain compounds that inhibit the proteolytic enzyme trypsin (Read and Haas, 1938) and the trypsin inhibitor is inactivated by heat treatment of soyabean meal.

However this heat treatment must be carefully controlled because overheating can result in the deterioration of the protein quality (see later).

The next stage for the process of digestion to occur, is the ability of the gastrointestinal tract to absorb the products of the enzymatic reactions. Inhibited transport to and passage of the gut wall (Friedrich, 1989) can be caused by the presence of tannins which form undigestible complexes with the dietary protein. Tannins are complex polymeric phenols present in many plants and their ingestion has been implicated in the decreased digestibility of dietary protein by chicks (Vohra *et al.*, 1966). According to McLeod (1974) the ingested tannins bind with proteins in the gastrointestinal tract resulting in the formation of compounds with bonds resistant to the digestive enzymes.

The nutritive value of the protein supplement can be markedly influenced by the method used in processing the protein supplement. Even though the availability of the amino acids can be improved by heat treatment of the food, i.e. the destruction of the heat labile proteolytic enzyme inhibitor in soya beans increasing the amounts of available amino acids, there can also be a negative effect from excessive heat treatment for long periods of time. In this

case interactions between functional groups within the protein or with other feed components like reducing sugars may occur. The most important reaction making an amino acid unavailable is the so called Maillard reaction. In the case of proteins mainly lysine is involved in these browning reactions since lysine can react with its free and reactive amino groups thus forming an unavailable lysine-sugar complex leading to further destruction of the lysine molecule. Many of these carbohydrate-amino acid complexes can be absorbed by the intestine but are not utilised for protein synthesis (Erbersdobler *et al.*, 1981). Lysine appears to be the amino acid most affected but the digestibilities of tryptophan, histidine and arginine can also be lowered.

Possibilities to improve protein digestibility are found in the use of process technology, biotechnology (enzymes, germination) and plant breeding. The effects of process technology, e.g. fractionation, autoclaving steam heating extrusion and toasting on the reduction of ANF and animal performance have been extensively studied (Van der Poel, 1990).

The supplementation of diets with exogenous enzymes can improve body gain and feed conversion in poultry. Only little information is available on the

possibilities to improve nitrogen digestibility and utilisation. Some studies indicate an improved nitrogen digestibility in broilers (Almiral *et al.*, 1993) and improved nitrogen retention in broilers (Samarasinghe and Wenk, 1993) with the inclusion of mixed enzymes in the diet. However, more research is required to evaluate the possible contribution of enzymes to reduce nitrogen excretion. Also apparent negative effects have to be taken into account when the use of enzyme supplementation for reduced nitrogen excretion is evaluated.

3.2.2 Endogenous Losses

As the food passes down the gut, especially as it enters the duodenum the nitrogen from the diet is diluted by endogenous nitrogen. This protein arises from several sources. Important sources include, saliva, gastric secretions, bile, pancreatic secretions and the desquamation of cells from the intestinal mucosa. Secretion of plasma proteins into the gut has also been suggested. From a nutritional point of view various problems concern the endogenous nitrogen such as the quantity secreted, its composition and its eventual utilisation.

Part of the endogenous nitrogen secreted into the lumen of the gastrointestinal

tract is digested and reabsorbed before the end of the ileum. This reabsorption has been estimated as 73% by Krawielitki *et al.*, (1990). Absorbed amino acids have to be used for the resynthesis of these endogenous secretions and estimates of efficiency range from 50-100% (Rohr and Lebzien, 1991). Consequently a considerable amount of nitrogen is lost and will be excreted in the urine.

3.2.3 Efficiency of Protein Utilisation

Once the nitrogen compounds are absorbed from the digestive tract into the chick's body the nitrogen losses are caused by poor utilisation of the absorbed protein. Maximum utilisation of dietary nitrogen requires a balanced ratio between the supplied amino acids, an optimal ratio between amino acid and energy supply, protein supply within the animal's genetic capacity and more efficient protein turnover.

3.2.3.1 Amino acid imbalance

Amino acid imbalance may be defined as a change in the pattern of amino acids in the diet resulting in depressions in food intake and growth which can be alleviated by supplementation with the first limiting amino acid (Harper, 1964).

Maximum utilisation of dietary nitrogen requires a balanced ratio between the supplied amino acids and much research has been conducted to determine the optimal ratio between the first limiting amino acids in poultry. In most practical diets there will be a mismatch between requirement and dietary supply for most amino acids. All amino acids constituting a protein to be synthesised must be available at the protein synthesis site simultaneously. If one amino acid is missing the synthesis of the whole protein is blocked resulting in the excess of all the other amino acids. Therefore, the other amino acids will be directed to other pathways resulting in increased amino acid degradation therefore an increase in nitrogen excretion. This brings in the concept of bioavailability.

In 1987, Sibbald used the word bioavailable to describe the proportion of an ingested nutrient which can be used for normal metabolic functions. The bioavailable amino acids are those which are actually supplied at the site of protein synthesis. If the dietary amino acid composition is adjusted in a way to supply exactly the required amount of the separate amino acids then there will be no excess amino acid supply to contribute to the nitrogen excretion. Therefore, nitrogen efficiency will improve.

One method of determining amino acid requirements is the diet dilution technique (Section 4.2) and this method has been adapted to investigate the growth responses of broiler chicks to different concentrations of indispensable amino acids (Gous, 1980; Morris *et al.*, 1987; Abebe and Morris, 1990a, b).

3.2.3.2 Body Protein Turnover

Proteins in the organs and tissues are continuously degraded and resynthesised in a process known as turnover. Increased protein supply stimulates protein synthesis and degradation (Waterlow *et al.*, 1978) which will in turn increase the rate of protein turnover.

Part of the inefficiency with which amino acids are utilised is a consequence of the turnover of proteins. The reutilisation of amino acids released on protein catabolism is not 100% efficient, as amino acids may be modified e.g. methylated and therefore are not available for the reincorporation into protein. The amino acids may also be removed from the turnover process for non protein purposes such as neurotransmitter synthesis. Increasing the rate of protein turnover would therefore increase the inefficiency of protein metabolism. However, this inefficiency may not be directly related to the

extent of protein turnover. The major determinant of the extent of protein degradation will be the concentration of amino acid in the amino acid pool associated with the amino acid catabolising enzymes.

3.2.4 Dietary protein catabolism

After absorption from the gastrointestinal tract, the major fate of dietary amino acids is incorporation into body protein or catabolism to carbon dioxide and uric acid. It is the metabolic partitioning between these two routes that has implications for the livestock industry. Most amino acids are stored in the body in the form of protein, the main store being the muscle and the remainder exist both intra and extra cellularly in the free amino acid pool.

When the amino acid is below the amount needed for maximal protein synthesis it is used with a relatively high efficiency (Beckett *et al.*, 1992). However amino acid degradation is a constant process as amino acid oxidation enzyme activity cannot be reduced to zero (Anon, 1990). It is this which allows protein turnover and remodeling to continue even at low protein intakes.

When the amino acid supply is in excess of requirements the increase in

amino acid oxidation for most amino acids tends to be proportional to the level of the amino acid in the diet (Gahl *et al.*, 1991). This has been observed for amino acids including lysine (Soliman and Harper, 1971. Brookes *et al.*, 1972), threonine (Kang-Lee and Harper, 1978), histidine (Kang-Lee and Harper, 1979; Kim *et al.*, 1983) and phenylalanine (Kim *et al.*, 1983). As supply exceeds demand the efficiency of utilisation for protein synthesis decreases and in order to maintain the tightly regulated free amino acid pool (Waterlow *et al.*, 1978) the carbon skeletons are used for alternative purposes such as lipogenesis or gluconeogenesis or oxidised to CO₂. This ensures that when amino acids are in short supply they are preferentially used for body protein synthesis. Therefore the primary role of amino acid catabolism is the removal of amino acids excess to requirement (Beckett *et al.*, 1992).

The first step in amino acid oxidation is the transamination of the α amino group resulting in the formation of the keto acid and ammonia. As ammonia is very toxic to the animal it is converted to uric acid via the uric acid cycle (Section 3.2.5.2).

The degradative pathways of many amino acids and the factors which effect them have been elucidated by in vivo oxidation studies which rely on the

collection of the expired CO₂. Such amino acids include threonine, histidine, phenylalanine and lysine. The increase in amino acid oxidation as the supply of amino acids exceeds the requirement for protein synthesis has also been used to assess the requirement of the animal to an individual amino acid (Section 4.2).

3.2.4.1 Lysine degradation

One of the amino acids which is of great interest in this experiment is lysine due to it being one of the most limiting amino acids in broiler diets and one which may be used as the reference amino acid to which all other indispensable amino acids are ratioed in the ideal pattern.

Accurate requirement estimates for digestible lysine are critical in attempts to apply the ideal protein concept in formulating broiler diets (Baker and Han, 1994) and extensive work has been done to estimate lysine requirements of broiler chicks (Han and Baker, 1991,1993).

In mammals lysine may be catabolised by two different pathways, the pipecolic and the saccharopinic. The pipecolic pathway proposed by Rothstein and Miller (1954) which involves pipecolic acid as an intermediate

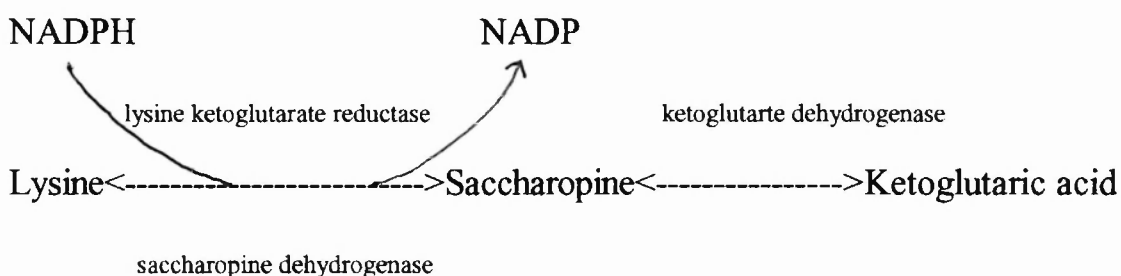
in the metabolic conversion of lysine to ketoglutarate has been found to be inert in rats and does not lie in the metabolic degradative pathway of lysine.

Lysine is catabolised in the liver to form α -ketoglutarate via the stable intermediate saccharopine (Figure 3.2). It was in 1965 that Higashino *et al.* suggested that saccharopine may be a key intermediate in the degradative pathway of lysine in rats and were the first to imply that the liver mitochondrion was the sole subcellular component able to convert lysine to CO_2 . It was during this study that isolated rat liver mitochondria were found to convert L-lysine to saccharopine in the presence of ketoglutarate. The enzyme catalysing the reaction was purified from rat liver mitochondria. (Noda and Ichihara, 1978). When investigating the initial degradative step in humans, the conversion of L-lysine to saccharopine was found to require the action of the catabolic enzyme lysine alpha ketoglutarate reductase (LKGR, EC 1.5.1.8) requiring the presence of NADPH (Hutzler and Dancis, 1968).

Grove and Roghair (1971) suggested that it is the saccharopinic pathway which is the major pathway for lysine degradation in the chick. Wang and Nesheim (1972) when studying lysine degradation in chicks found that two reaction schemes were available in chick liver, but that the saccharopinic was

more significant. They also suggested that lysine ketoglutarate and NADPH availability may play a significant role in the regulation of lysine degradation in chicks. Ketoglutarate is considered as a cofactor for lysine ketoglutarate reductase.

Figure 3.2: The saccharopinic pathway towards lysine degradation



Following studies with rats Blemings *et al.* (1994) stated that this enzyme was found in the mitochondrial matrix. It was this observation that suggested that an understanding of the factors affecting lysine entry into the matrix could be central to the understanding of regulation of lysine catabolism if it could show that transport limits lysine degradation.

It is well known that the feeding of high protein diets produces an increase in the activities of numerous liver enzymes involved in the catabolism of

amino acids (Soliman and Harper, 1971; Ip and Harper, 1973; Kang-Lee and Harper, 1978), but little is known about dietary effects on the activity of LKR. More recent work includes the dietary effects of lysine on lysine catabolism in rats.

Little research has been done regarding the study of lysine catabolism in the growing chick, especially concerning the activity of the enzyme lysine ketoglutarate reductase. Due to lysine being the limiting amino acid for protein synthesis in poultry an understanding of the mechanisms that regulate LKGR activity is of nutritional importance because any unnecessary lysine oxidation is clearly wasteful.

A more thorough understanding of factors controlling lysine utilisation is of economic importance in food animal production and of medical importance with respect to the use of low protein diets by humans.

3.2.4.2 Uric acid

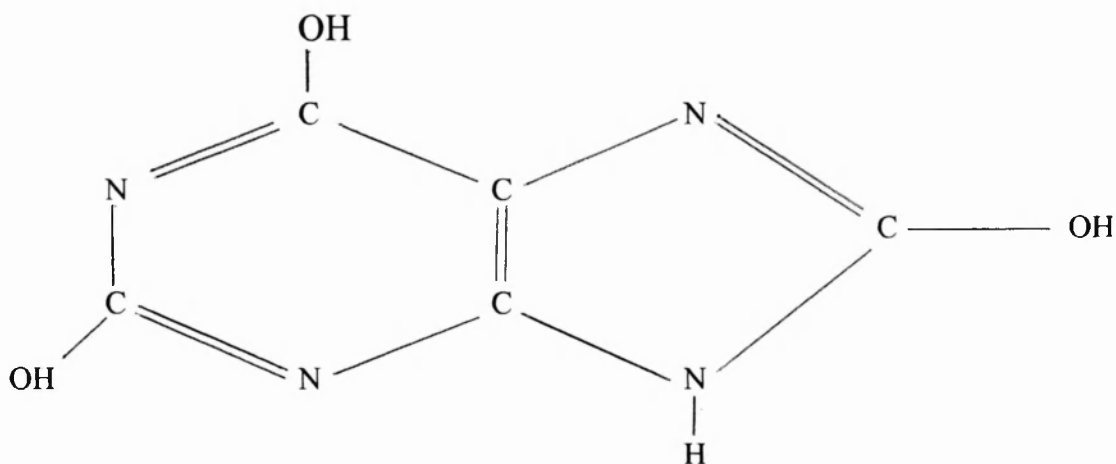
As mentioned earlier one of the end products of amino acid metabolism is uric acid. Urine nitrogen excretion increases linearly with nitrogen intake and uric acid accounts for over 80% of urinary nitrogen in the chick (McNabb and

McNabb, 1974).

Miles and Featherstone (1974) have shown how uric acid excretion can be used to predict the requirement for amino acids in the growing chick. Their data also suggests that the concentration of uric acid in plasma also has potential value as an indicator of amino acid requirements.

Estimates of protein digestibility and the protein requirements of poultry demand a knowledge of the composition and amount of nitrogen which is excreted. There have been a number of investigations into the nitrogenous fraction of poultry urine (Miles and Featherstone, 1974) resulting in the observation that the major excretory product of nitrogen metabolism in birds is uric acid. Uric acid is synthesised in the liver and the kidney excretes of poultry by means of filtration and tubular secretion, the uric acid presented to it through the vascular system.

Fig 3.3: The structure of uric acid



The pathway of biosynthesis of uric acid has been clarified and the metabolic precursors of the nitrogen in the purine ring have been identified. Two of the nitrogen atoms arise from the amide nitrogen of glutamine, the other two nitrogen arising from the amino nitrogen of glycine and aspartate. Glutamine is synthesised from glutamate and ammonia arising from the deamination of amino acids. It is the nitrogen from ammonia that appears in uric acid. Glutamate acts as a carrier form of ammonia and in addition to providing ammonia for purine synthesis, glutamine synthesis can prevent accumulation of ammonia.

The cloaca of poultry combines the function of the rectal ampulla and urine bladder as formed in mammalian species into one (Moran *et al.*, 1982) and the voiding involves cloacal contraction that moves all faeces and urine out together, which therefore handicaps the studies on protein metabolism. Since approximately 80% of urinary nitrogen is present as uric acid, separation of uric acid nitrogen in poultry excreta is crucial for nitrogen studies.

The procedure currently used for the uric acid analysis of biological fluids involves enzymatic degradation by uricase. Complete extraction of uric acid from excreta is one of the most critical steps in the determination of uric acid.

3.2.5 Theories Behind Protein Synthesis and Amino Acid Oxidation

As mentioned previously in this chapter, protein synthesis in poultry increases to a maximum point above which no further increases are observed. At the same time there is an increase in amino acid oxidation resulting in the excess amino acids above requirement being excreted as nitrogen, hence the cause of nitrogen pollution.

Mechanisms involved in the process of protein synthesis are well documented (Reeds and Davis, 1992) but there are few reports concerning the factors

controlling amino acid catabolism which results in the excess nitrogen excretion, especially in relation to farm species. Poultry operations can be potential source of nitrogen pollution. This has been confirmed by nitrogen balance studies which have shown that with an increase in nitrogen intake, there is an increase in nitrogen retention and excretion. (Carr *et al.*, 1977). At higher nitrogen intakes however nitrogen excretion increases relative to nitrogen retention in that the efficiency of nitrogen metabolism is lowered. The response of nitrogen retention to an increase in nitrogen intake (Dunkin *et al.*, 1986) is similar to the response of protein synthesis to increasing amino acid supply but any relationship between protein breakdown and uric acid excretion is dependent on the rate of amino acid catabolism and uric acid cycle activity.

Both protein synthesis and amino acid oxidation are responsive to amino acid supply (Waterlow *et al.*, 1978; Brookes *et al.*, 1972). The K_m values of the enzymes involved in the catabolism of amino acids are higher than those of the amino acid activating enzymes (Kang-Lee and Harper, 1978). At amino acid concentrations below requirement an increase in amino acid supply results in stimulation of protein synthesis while amino acid oxidation is unaffected. At higher amino acid concentrations an increased amino acid

supply has less effect on protein synthesis but amino acid oxidation is enhanced. This suggests that at low amino acid concentrations as a consequence of reduced amino acid supply amino acids are preferentially used for protein synthesis but when the supply is adequate or in excess of requirements and maximal rates of protein synthesis are obtained the remaining amino acids are catabolised, thereby increasing nitrogen excretion.

In 1988, Millward and Rivers developed the anabolic drive hypothesis which implied a connection between amino acid oxidation and protein synthesis. They stated that with an increase in amino acid oxidation due to an increased supply, there will be an increase in protein synthesis and that before their oxidation, excesses of amino acids exert a transient anabolic effect on protein deposition. Therefore rapid growth as a consequence of high protein diets, may result in increased amino acid degradation hence increased nitrogen excretion.

It is often assumed that amino acid oxidation is an inescapable consequence of dietary amino acid supply, with the catabolism in the liver functioning to prevent potentially toxic concentrations of amino acids reaching the peripheral circulation. However, in a series of in vivo experiments with rats

Ip and Harper (1973) studied the effect of different protein levels (6% or 24% casein) and a tyrosine concentration maintained at 50g/kg on amino acid oxidation. It was shown that the high protein diet enhanced the activity of the liver tyrosine catabolic system because the tissue tyrosine concentration was lower. Therefore an enhanced supply of protein was associated with an increase in the capacity to catabolise the amino acid whose supply was maintained at a constant level.

This apparent increase in amino acid oxidation associated with an increase in protein supply would account for some of the inefficiency in nitrogen metabolism in animals.

Another theory behind protein synthesis is that the increase in amino acid oxidation could simply be due to increased cycling of protein (Hawkey, 1994). Amino acids are continually recycled. Amino acids resulting from protein breakdown may enter the free pool or be directly used for protein synthesis (Barnes *et al.*, 1992) whilst amino acids in the free pool may be removed for incorporation into protein. Increased protein supply stimulates protein synthesis and degradation (Waterlow *et al.*, 1978) which will in turn increase the rate of protein turnover. This cycling is not 100% efficient as

some may be modified and therefore not available for incorporation into protein. Also, they may be removed from the cycle for non protein purposes. Increasing the rate of protein turnover would therefore increase the inefficiency of protein metabolism. Compartmentalisation may also effect turnover.

If there is a link between protein synthesis and amino acid oxidation then it follows that enhanced protein deposition will be associated with an enhanced rate of amino acid catabolism which particularly in livestock production could result in increased environmental pollution.

CHAPTER 4

AMINO ACID REQUIREMENTS OF POULTRY

4.0 Amino acid requirements of growing poultry

4.1 Amino acids in the diet

The provision of amino acids either in free form or in the form of protein is believed to account for approximately one quarter of the cost of practical poultry diets (McNab, 1994). However, to consider administering an amino acid supply lower than required there can be a substantial impairment in productivity therefore resulting in a much greater influence. Therefore an important objective of animal nutrition is the formulation of poultry diets which are able to allow a rate of production to be achieved but at the least economical cost, which suggests that the diet must satisfy the requirements of the species for amino acids. It is unlikely that the maximum economic return in poultry production can be achieved unless the amino acid concentrations in the diets are known to meet the requirements of the animals being fed (McNab, 1994).

The contribution made by dietary protein to the nutritional needs of the animal depends not only on its amino acid composition but also how effectively the amino acids are utilised. Lists of amino acid components of feedstuffs used in poultry diets are regularly published (NRC, 1994).

However these figures are only useful in predicting the maximum value of the protein and the contribution made by these amino acids may fall short of the maximum. The reason for this is that not all of the amino acids in the dietary protein become available to the animal during digestion and metabolism.

It is known that in poultry production there are relatively high food costs and small profit margins therefore resulting in considerable pressure to reduce the extent of over formulation of price sensitive nutrients. This over formulation is also the cause of the environmental consequences of intensive animal production due to the excess amino acids being excreted into the environment therefore the brunt of mounting criticism with regards to environmental pollution. The excretion of these excess amino acids can also cause welfare problems.

4.2 Methods of determining amino acid responses

There are several techniques used to determine amino acid responses in growing poultry. One of the most frequently used approaches involves a basal diet lacking in one or more amino acids to which the limiting amino acid under test is stepwise supplemented up to a maximal response level

(D'Mello, 1982). This is known as the graded supplementation technique and the prerequisites for such a method are that the basal diet used must be deficient in the amino acid under test. The graded doses of the test amino acid must be formulated to generate a full response curve including doses which are well below the animals requirement and doses which produce a maximum response. The requirement of food intake is also an essential prerequisite for interpretation of the data. It is at the optimum point on the response curve where performance is no longer improved that can be taken as the amino acid requirement for the response parameter in question. The curve may also be used to determine estimates of slope and plateau values required as important components for interpretation by the Reading model (Fisher *et al.*, 1973).

It was in 1970 while studying the response of laying hens to increasing intakes of methionine, that Fisher and Morris developed the sequential dilution of a 'summit' diet. This procedure involves the sequential dilution of a high protein (summit) diet with an isoenergetic protein free mixture, which relies on an amino acid imbalance to determine optimum amino acid requirements. When the blending takes place, the amino acid under test will be first limiting at all dilution levels. Although the dilutions result in different

protein concentrations, the dietary amino acid pattern remains constant throughout the diluted series. At each level of dilution, supplementation with the pure form of the limiting amino acid may be undertaken, which should elicit responses that are compatible with those obtained by diluting the summit diet. It is this method of dilution and supplementation which was adopted by Gous (1980), Morris *et al.* (1987) and Abebe and Morris (1990a,b) when studying the growth responses of broiler chicks to different concentrations of indispensable amino acid.

It is with both methods however that much criticism has been observed. Such criticisms include those by Fisher and Morris, (1970) and Gous, (1980) who claimed that with respect to the supplementation technique there are a number of disadvantages involved with this method. Such disadvantages include that the response may be influenced by the fact that the dietary amino acid balance changes with each successive dose of the limiting amino acid. It has also been argued that at high levels of supplementation of the amino acid under test may no longer be first limiting and that the responses may be prevented by the second limiting amino acid. It is also thought to be difficult to obtain a satisfactory basal diet sufficiently deficient to allow the use of a wide range of input levels of the amino acid. Another disadvantage was

thought to be the economical cost of using synthetic amino acids which may result in the restriction of research work into amino acid responses.

However none of these apply to the diet dilution technique and it is thought that the above disadvantages may be overcome by using the diet dilution technique. Fisher and Morris (1970) claimed the technique satisfied all the requirements for a successful assay and Gous (1980,1986) described it as an improved method.

It was in 1982 however that D'Mello concluded that the lack of confidence in the responses from the graded supplementation technique and the diet dilution technique as an improved method could not be justified since both methods yielded concordant growth. This was also in agreement with Boorman and Burgess (1986) who arrived at similar conclusions after studies on lysine responses in broiler chicks.

Since these reviews however a feature of the diet dilution technique has been observed which renews the doubt of earlier attempts to validate the technique (Gous 1980; Gous and Morris 1985). It is thought that the method relies on the interpretation of responses to different rates of dilution as

responses to the first limiting amino acid and not to the change in dietary protein contents. However it was work by Morris *et al.* (1987) and Abebe and Morris (1990a,b) that demonstrated that crude protein level does influence the growth response to an amino acid. A reassessment from D'Mello, showed that the growth response obtained by dilution was quite distinct from that obtained on supplementation of each diluted diet with pure lysine.

Although much criticism has been observed the graded supplementation procedure still remains the method of choice in the majority of studies on amino acids in growing poultry. However, because growth is affected by many complex factors than the amino acid balance, many physiological responses have been used to assess the amino acid requirement.

Brookes *et al.* (1972) used the amino acid oxidation technique to assess the lysine requirement of the rat. This was performed by providing an excess of the amino acid under study and assessing the oxidation rate. The point whereby the oxidation rate increased known as a 'break point' or 'inflection point' provided a means for estimating the requirement. This concept was further used for the determination of lysine requirement of young pigs

(Chavez and Bayley, 1976), histidine in rats (Kang-Lee & Harper, 1977) and histidine and phenylalanine in pigs (Kim *et al.*, 1983). All these studies used the collection of expired CO₂ after the administration of labeled amino acids.

4.3 Statistical evaluation of amino acid requirements.

The statistical model used to determine the requirement of an amino acid is either known as a bent stick model or an exponential curve showing the relationship between amino acid intake and performance. Theoretically one would expect a bent stick but in practice a smooth curve is observed.

Fisher *et al.* (1973) explained that the integration of several different broken lines within a population slightly differing in response will lead to a smooth curve. Therefore implying that with each determination of requirements slightly different results may be expected because the transition point is not a sharp point but more an area. Whether a requirement value should be taken very strictly or not depends on the change in response when values just above or under the indicated requirement are fed. Also, the steepness of the response curve against dietary intake level and how it behaves after the transition point. Due to this, requirement values should be presented with some kind of sensitivity parameter. Requirement values found might depend

on the response parameters used. Fisher (1981) and Clark, Gous and Morris (1982) have used this model to describe the responses of growing birds to amino acid intake.

A parabolic curve will often give a good fit to experimental data, however the shape of a fitted parabolic curve is sensitive to the range of the treatments selected. One difficulty is that it predicts a reduction of output beyond the maximum dose. Many nutrients do cause a reduction in output when fed in excess but the response curve is seldom symmetrical. The method used by Morris *et al.* (1987) to determine optimum lysine concentration was performed by fitting a quadratic equation to data relating growth rate to dietary lysine content at different protein concentrations. The maximum point was calculated resulting in estimates of optimum lysine concentrations however this data was thought to be influenced by the range of treatments chosen for the experiment (Morris, 1983).

This methodology of estimating a requirement for lysine is open to criticism because quadratic curves do not always represent the data adequately and a more optimum input is more defensible than the requirement for growth. However alternative methods of analysis were described by Abebe and

Morris (1990) and both methods were applied without changing the conclusions in any way. The two procedures used for estimating optimum dietary lysine concentrations were using a parabolic procedure and the Reading Model (Fisher *et al.*, 1973). The Reading model was chosen in preference due to its curvature being largely independent of the range and spacing of the experimental treatments. However although the methods produced different estimates of requirement for example the estimates of lysine requirement tended to be lower when based on 0.97 of maximum growth rate in a Reading model, the estimated lysine requirement was still a function of the dietary protein concentration.

4.4 Estimation of Amino Acid Requirements

An amino acid requirement may be defined as the amount of amino acid required relative to other amino acids that a bird should receive depending on the response parameter to be assessed. Requirement values found might depend on the response parameters used as shown by Heger and Frydrich (1989) when it was found that the requirement for maximum nitrogen efficiency may be lower than the requirement for maximum production. In studies of the amino acid requirements of the growing chick, weight gain, efficiency of food utilisation and nitrogen retention are the parameters that are

more commonly used.

There has been much debate as to what is the most appropriate way to express the dietary requirement of the animal for amino acids. One way is to consider amino acids in poultry in relation to dietary concentrations (e.g. g/kg diet). Another way is to express requirements as a proportion of dietary crude protein (Barbour *et al.*, 1993). It is well established (Grau, 1948; Almquist, 1952) that when protein limiting diets are fed to chicks the optimal concentration of the first limiting amino acid depends on the supply of the second limiting amino acid. It is for this reason that the requirement for a limiting amino acid such as lysine increases in direct proportion to the protein content of the diet provided that its protein composition is held constant and the overall supply remains limiting. The use of intake rather than dietary concentrations to interpret responses of poultry to the first limiting amino acid has long been advocated by Morris (1972,1983). However in a further study Morris *et al.* (1987) rejected this approach and used dietary concentrations to consider the response of broiler chicks to lysine and protein. Morris *et al.* (1987) have further shown that the lysine required for maximum growth or maximum efficiency of food utilisation continues to increase in direct proportion to the dietary protein concentration even in the range where no

additional growth response was obtained.

They concluded that a fixed ratio of lysine to protein should be specified in practical diet formulation rather than a minimum dietary concentration of lysine. This would ensure that if the dietary protein content rises above a prescribed minimum value in least cost formulation an appropriate adjustment will automatically be made to the lysine content of the solution.

It was found that when diets varying in crude protein from 140 to 280 g/kg were fed to growing chicks the amount of lysine needed to maximise the growth rate at each protein concentration was a fixed proportion of that protein which was approximately 54 g/kg CP. This rule has been well established for diets with limiting protein contents and can be explained by the hypothesis that as the supply of the first limiting amino acid is increased the limit to the response is determined by the supply of the second limiting amino acid (as stated above). The implication of this observation is that any surplus protein in the chick diet has an adverse effect on the utilisation of the first limiting amino acid.

In a paper by Morris and Abebe (1990) whereby the effect of arginine and

protein on chicks responses to lysine were studied, the estimates of lysine requirement were 56g/kg CP for maximum growth or 56g/kg for maximum efficiency, whereas the lysine requirements by Morris, 1987 were estimated at 53g/kg CP and 55g/kg CP. The differences may have represented experimental error or may have reflected the balance of the amino acids in the protein mixtures used (Morris and Abebe, 1990).

CHAPTER 5

THE EFFECT OF DIFFERENT LEVELS OF PROTEIN SUPPLY ON NITROGEN UTILISATION IN GROWING CHICKS

5.1 OBJECTIVES

The objective of this experiment was to quantify the efficiency of nitrogen retention of broiler chickens given diets containing eight different levels of protein. The protein in all diets was approximately equal to the ideal essential amino acid concentration for this age of broiler chicken.

5.2 MATERIALS AND METHODS

5.2.1 Treatments

Eight dietary treatments were formulated that contained either 130, 155, 175, 190, 215, 240, 270 or 300g/kg diet of crude protein. Diets were otherwise similar in metabolisable energy, calcium, phosphorus, linoleic acid, vitamin concentrations and amino acid balance. The increasing protein concentration was achieved by adding poultry offal in replacement for soyabean meal, maize and fishmeal (Table 5.1).

5.2.2 Bird Management

One hundred and sixty female Cobb hybrid broiler chickens were group housed in a light and temperature controlled room until 28 days of age. A proprietary diet and water was provided *ad lib*. At the start of the experimental period, 96 birds were weighed and placed in cages (two birds per cage). The cages (37cm x 37cm) were in a controlled environment room with light at 23 hours per day, temperature at 24°C and a relative humidity of 85% (\pm 5%). Food and water was provided with free access.

A seven day feeding period was given. On day 0 and day 7 the birds were

weighed per cage and feed intakes were measured for the first 4 and last 3 days of the feeding period. This was performed by providing each cage with its own feed bag and weighing the bag over time.

Excreta was collected on the last 4 days of the feeding period. The excreta was removed from every tray after each 24 hour period and mixed using a food blender. The excreta was frozen until required for nitrogen determination.

Table 5.1. Experimental diets

Ingredients	Crude Protein Concentrations (g/kg diet)							
	130	155	172	198	215	240	274	300
Maize	819	778	751	710	683	643	589	548
Soyabean meal	6	9	11	14	16	18	22	25
Fishmeal	5	7	9	11	12	15	18	20
Poultry Offal	90	133	162	206	235	278	336	380
Limestone	5	4.3	3.7	3	2.5	1.8	0.8	0
Dicalciumphosphate	40	35	31	26	23	17	10	5
Salt	3	2.6	2.3	1.8	1.5	1.1	0.5	0
Vitamin/Mineral Premix	20	20	20	20	20	20	20	20
Soya Oil	10	8.5	7.5	6	5	3.5	1.5	0
Lysine	1.5	1.3	1.1	1.0	1.3	0.7	0.4	0.2
Tryptophan	0.3	0.3	0.4	0.4	0.4	0.4	0.5	0.5
Threonine	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Methionine	0	0.1	0.1	0.2	0.2	0.3	0.3	0.4
<u>Calculated composition</u>								
ME (MJ/kg)	13.13	13.15	13.16	13.17	13.19	13.20	13.22	13.24
Crude Protein (g/kg diet)	129.7	155.2	172.2	197.7	214.7	240.2	274.1	299.6
Lysine (g/kg CP)	50	50	50	50	50	50	50	50
Methionine/Cystine (g/kg CP)	38	38	38	37	37	37	36	36
Threonine (g/kg CP)	37	37	37	37	37	37	37	37
Tryptophan (g/kg CP)	9	9	9	9	9	9	9	9

5.2.3 Sample analysis

5.2.3.1 General chemicals

All the chemicals used in the following methods were of analytical grade unless otherwise stated and were obtained from either Sigma Chemical Co., Poole, Dorset, or BDH Chemicals Ltd., Poole, Dorset. All water used in the following methods was deionised and all glassware underwent a strict cleaning regime.

5.2.3.2 Nitrogen analysis

The nitrogen content of the feedstuffs and excreta samples were determined using the Kjeldahl procedure (AOAC method number 954.01). For feedstuff analysis, approximately 0.5 grams of feed was used. For excreta analysis, approximately 3 grams of fresh excreta was used.

5.2.3.3 Uric acid analysis

This method is a modification of that according to Pudalkiewicz *et al.* (1967) and is used in this experiment to analyse the uric acid content of fresh excreta. A number of months were spent modifying this procedure to ensure the reproducibility of using fresh excreta as a time saving method. Therefore

the procedure will be explained in full.

All glassware used in the following procedures underwent a strict cleaning regime which consisted of soaking in 5% Decon 90 solution followed by an acid wash in 0.1M HCl followed by thorough rinsing in deionised water and drying in an oven.

5.2.3.3.1 Reagent Preparation

Lithium Carbonate (LiCO_2) 0.5%: 5 grams of Lithium Carbonate (Sigma L-3876) was dissolved in deionised water and made up to volume in a 1 litre volumetric flask.

Glycine buffer (0.1M, pH 9.2 ± 0.1): 7.56g of Glycine (Sigma G-7403) was dissolved in 800 ml of deionised water. The solution was adjusted to the desired pH using 10N NaOH and made up to volume in a 1 litre volumetric flask.

Uricase: The uricase used in the present study was obtained commercially and depending on the activity an appropriate amount was dissolved in 0.1M Glycine buffer. 0.04g of uricase ((E.C.1.7.3.3 (Sigma U-3500)) was dissolved

in 1000ml of 0.1M glycine buffer according to Dubbs *et al.*, 1955. The preparation was stable for approximately one month when stored in the refrigerator.

5.2.3.3.2 Uric acid standard preparation

The uric acid standard stock solution was made by dissolving 100mg of the purified uric acid (Sigma U-0881) in 12ml of 0.5% LiCO₂ at 60°C. The solution was then cooled and made up to volume in a 100ml volumetric flask using deionised water.

Serial dilutions of the standard stock solution were made with glycine buffer so as to give concentrations between 1 and 10µg per ml. One ml of the standard stock was added to a 100ml volumetric flask and made up to volume with glycine buffer to make a 10µg/ml standard solution. Serial dilutions were then made to make 1µg/ml (100µl standard solution plus 900µl buffer), 2µg/ml (200µl standard solution plus 800µl buffer), 4µg/ml (400µl standard solution plus 600µl buffer), 6µg/ml (600µl standard solution plus 400µl buffer), 8µg/ml (800µl standard solution plus 200µl buffer) and 10µg/ml (1ml standard solution). A standard curve was prepared by plotting absorbance at 292nm against concentration.

5.2.3.3.3 Sample analysis

One gram samples of fresh excreta were transferred to a 250ml volumetric flask and the neck of each flask was washed down with 50ml of 0.5% LiCO_2 solution. Samples were extracted for 30 minutes by frequent swirling at a speed reading of 120 on a HS 501 Digital Ikalabortechnik shaker. The flasks were then made up to volume with deionised water and mixed by inversion with the aid of parafilm. A 25ml portion of each sample was then centrifuged at 3500rpm to remove solids. 1 μl of each sample was added to 9 μl of glycine buffer to make a 1 in 10 dilution. A blank was prepared using 1ml of glycine buffer and 9ml uricase and mixed by inversion with the aid of parafilm.

Cell corrections for possible changes in absorbance of uricase solution were obtained by reading the blank solution at 292nm before and after incubation. Extinction readings on test samples were made by adding 9ml of uricase solution and mixing by inversion using parafilm. Samples were returned to the tubes following reading. After all the samples were read they were then incubated at 45°C for 4 hours. After incubation samples were again read following corrections using the incubated blank.

5.2.3.3.4 Uric acid calculations

Dry matter contents of each excreta sample were calculated by oven drying samples at 300°C to a constant weight. The amount of mg of uric acid per gram of excreta was then calculated using the following equation:

$$\frac{(E \text{ (initial)} - E \text{ (terminal)}) \times (\text{dilution factor})}{((k \times (\text{sample weight} \times \text{dry matter}) \times 1000))}$$

E (initial) = initial absorbance

E (terminal) = final absorbance

Dilution factor = 250 (volume of flask) X 10 (1 in 10 dilution of sample against buffer) X 10 (1 in 10 dilution of diluted sample against uricase) = 25,000.

k = change in extinction/μg of uric acid/ml (slope) and was obtained by the calibration curve.

Sample weight = ~1g

1000 = value required to convert μg to mg

According to Dubbs *et al* (1956) a k value of 0.073 is acceptable

5.2.4 Calculations

5.2.4.1 Feed conversion ratio

Feed conversion ratio was calculated by: weight gain/feed intake.

5.2.4.2 Nitrogen excreted as uric acid

33% of uric acid is in the form of nitrogen, therefore nitrogen excreted as uric acid was calculated by: $0.33 \times \text{total uric acid excreted}$.

5.2.4.3 Nitrogen excreted in the faeces

Of that nitrogen which is digestible, approximately 80% is excreted as uric acid (McNabb and McNabb, 1975) in the broiler chick. Therefore nitrogen excreted in the faeces may be calculated by: $\text{nitrogen excreted} - (\text{nitrogen excreted as uric acid} \times (100/81.4))$.

5.2.4.4 Protein digestibility

The digestibility of the feedstuff may be calculated by:

$((\text{Nitrogen Intake} - \text{Nitrogen excreted in the faeces})/\text{Nitrogen Intake})$.

5.2.4.5 Efficiency of Nitrogen Utilisation

The percentage nitrogen retention may be calculated by:

$$(\text{Nitrogen Intake} - \text{Nitrogen Excreted}) / (\text{Nitrogen Intake} \times \text{Digestibility}).$$

5.2.5 Statistical Analysis

Randomised block analysis of variance was performed. The treatment (level of dietary crude protein) sum of squares, were partitioned into their linear and non linear effects within the analysis of variance, using the Genstat computer package (Lawes Agricultural Trust 1990).

5.3 RESULTS

Dietary protein concentration had no significant effect on the weight gains and feed intakes of the broilers (Table: 5.2). However, increasing dietary protein concentrations resulted in increasing ($p<0.001$) intakes of nitrogen and increasing ($p<0.001$) nitrogen excretion (Table: 5.3).

There was no significant effect of diet on the amount of nitrogen retained in the body. Total uric acid excreted and nitrogen excreted in the urine increased ($p<0.001$) in response to an increase in dietary protein concentration (Table: 5.4). However increasing dietary protein concentration resulted in lower ($p<0.001$) protein digestibility and an increase ($p<0.001$) in the amount of nitrogen excreted in the faeces. Increasing dietary protein concentration resulted in a lower ($p<0.05$) percentage of digestible nitrogen being retained.

Table: 5.2 Effect of dietary protein on weight gain, feed intake and feed conversion efficiency.

	Dietary protein concentration (g/kg diet)								
	130	155	175	190	215	240	270	300	SEM (30 residual d.f.)
Weight gain (g/day)	-7.9	5.4	-6.7	5.2	9.8	0.5	-4.6	7.0	6.61
Feed intake (g/day)	142.9	142.0	143.3	125.8	132.1	122.5	110.0	135.6	12.08
Food Conversion Efficiency	-0.069	0.019	-0.051	0.055	0.056	-0.009	-0.071	0.049	0.0526

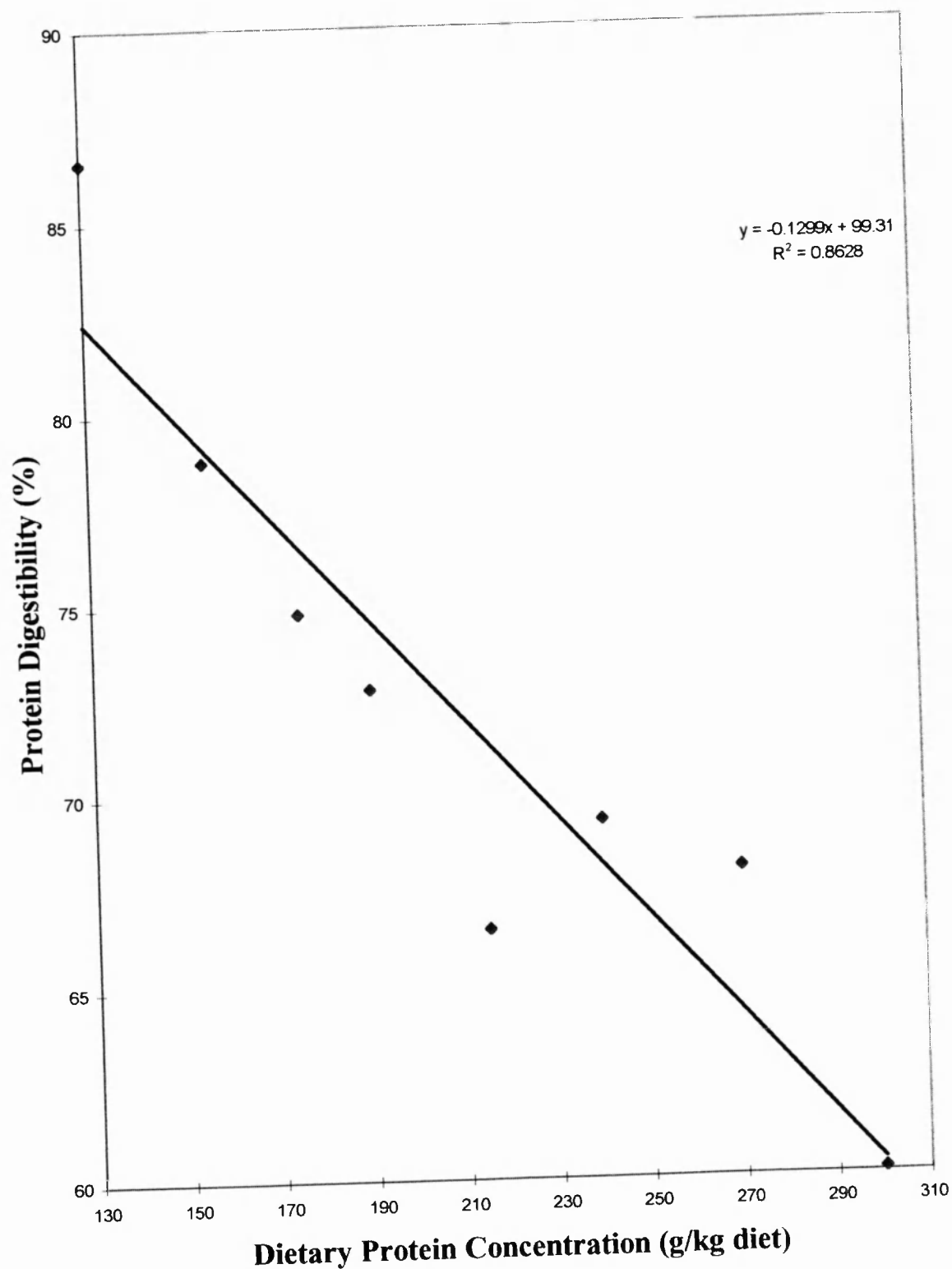
Table: 5.3 Effect of dietary protein on nitrogen intake, excretion, retention and retention as a proportion of nitrogen intake.

	Dietary protein concentration (g/kg diet)								
	130	155	175	190	215	240	270	300	SEM (30 residual d.f.)
Nitrogen intake (g/day)	3.15	3.15	3.99	4.13	4.61	5.23	5.04	7.22	1.682
Nitrogen excreted (g/day)	1.72	2.02	2.29	2.37	3.23	3.52	3.68	5.83	1.121
Nitrogen retention (g/day)	1.425	1.130	1.698	1.760	1.373	1.708	1.360	1.398	0.0384
Nitrogen Retention (%)	45.0	32.2	41.2	42.9	30.5	32.9	22.9	19.6	4.70

Table: 5.4 Effect of protein content on total uric acid, nitrogen excreted as uric acid, nitrogen excreted in the faeces, protein digestibility and percent of digestible nitrogen retained.

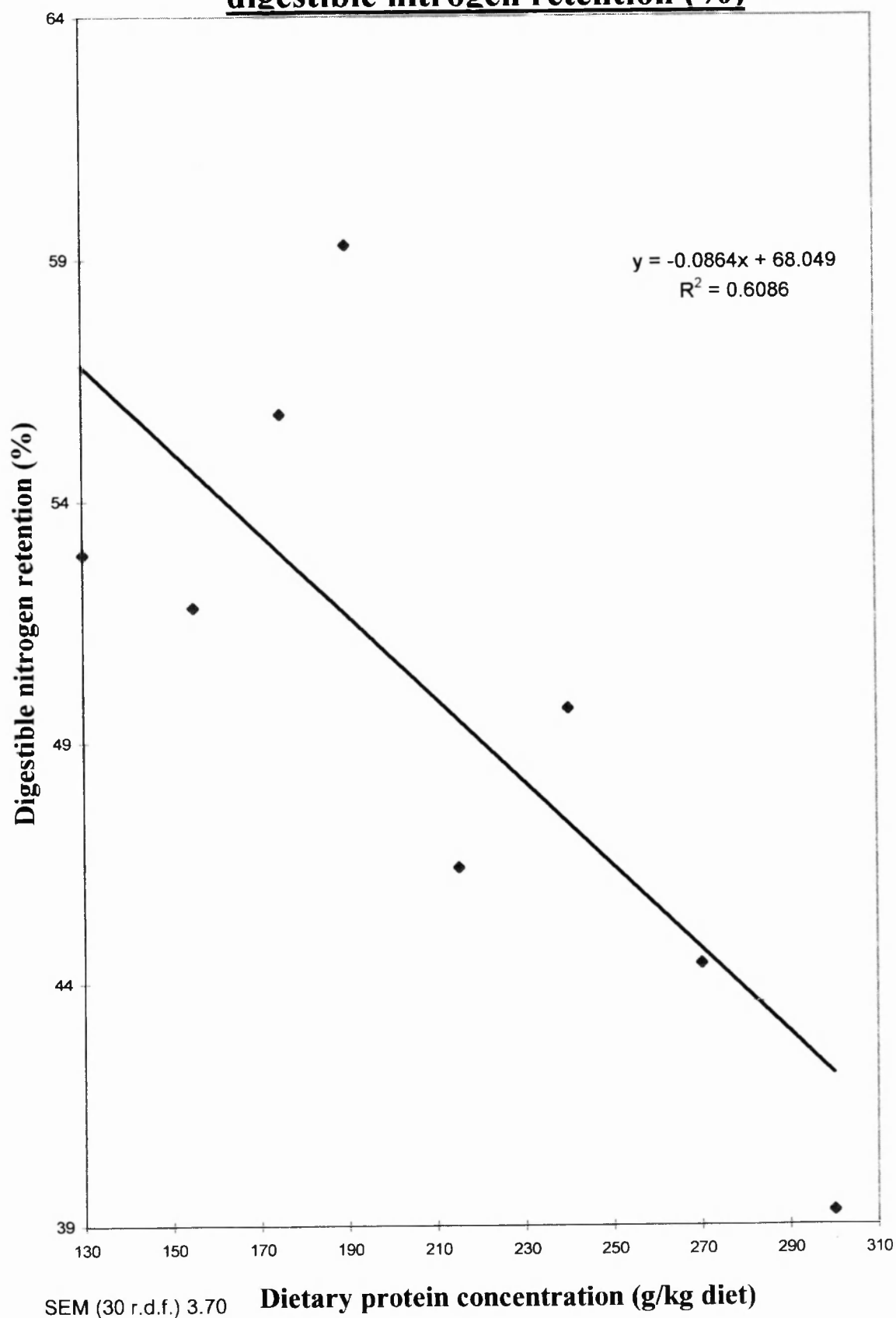
	Dietary protein concentration (g/kg diet)										SEM 30 residual d.f.
	130	155	175	190	215	240	270	300			
Nitrogen intake (g/day)	3.15	3.15	3.99	4.13	4.61	5.23	5.04	7.22			1.682
Total uric acid excreted (g/day)	3.16	3.13	3.32	3.11	4.20	4.81	4.77	6.46			0.534
Total nitrogen excreted in urine (g/day)	1.04	1.03	1.09	1.03	1.39	1.59	1.58	2.13			0.176
Total nitrogen in faeces (g/day)	0.44	0.82	0.95	1.11	1.53	1.57	1.67	2.79			0.205
Protein digestibility (%)	86.6	78.8	74.8	72.8	66.5	69.3	68.0	60.1			3.70
Digestible nitrogen intake (g/day)	2.72	2.49	2.99	3.01	3.06	3.62	3.43	4.34			0.544
Digestible nitrogen Retention (%)	52.9	51.8	55.8	59.3	46.4	49.7	44.4	39.3			3.70

**Fig 5.1: Effect of dietary protein concentration
on protein digestibility (%)**



SEM (30 r.d.f.) 3.70

Fig 5.2:Effect of dietary protein concentration on digestible nitrogen retention (%)



5.4 DISCUSSION

There was excessively low protein digestibility (Figure 5.1) that resulted in poor growth rates and no significant differences in nitrogen retention as the dietary protein concentration increased. The results from this experiment contradict previously published and expected responses to dietary protein supply. It is well established that increasing dietary protein concentration causes an increase in weight gain (Clark, Gous and Morris, 1982) and an increase in nitrogen retention to a maximal level until no further increases are observed (Carr *et al.*, 1977, Muramatsu *et al.*, 1987). The present experiment did not give results that were consistent with expected biological responses, so this experiment did not achieve its objectives.

There were two major problems; a decreasing protein digestibility with increasing dietary protein concentration (Figure 5.1) and a decreasing proportion of digestible nitrogen retained with increasing dietary protein concentration (Figure 5.2). The reasons for the unexpected results could possibly be due to the diet formulation and especially the major protein source, poultry offal. The poultry offal used in this experiment was aimed to provide a high protein diet with an ideal amino acid balance for the broiler

chick. The factor which may have affected the digestibility of the feedstuff is the heat applied during the feedstuff processing. This heat is necessary to reduce microbiological health risks. However excess heating may also reduce the protein quality due to decreased amino acid availability or digestibility (Parsons *et al.*, 1992). Excessive or prolonged heating during drying will lower the digestibility and cause some loss of essential amino acids. Excessive heat causes the formation of new enzyme resistant linkages within the protein molecule thereby reducing the digestibility and availability of the constituting amino acids. The poultry offal may have been severely heat treated thereby causing low digestibility.

The decreasing proportion of nitrogen retention may also have been a result of high processing temperatures. Batterham (1992) proposed that discrepancies between digestible and bioavailable values was probably due to the formation of early Maillard compounds during heating. Many of these carbohydrate-amino acid complexes can be absorbed in the intestine but are not utilisable for protein synthesis (Erbersdobler *et al.*, 1981) and are then often excreted unchanged in the urine (Hurrel , 1990). Although there was a significant increase in the amount of digestible nitrogen eaten in the treatments with increasing dietary protein concentrations, there was a

significant decrease in the percentage retention of this nitrogen. This indicates the digestible protein was not available for protein synthesis. It is likely that the digestible protein may have been affected by the result of reactions of protein with other components of the feedstuff. An example of this kind is the Maillard reaction whereby the amino groups from a free amino acid or an amino acid side chain on the protein combine with the carbonyl carbon of a reducing sugar to form a glucosyl amino compound.

Therefore in this experiment the formation of early Maillard compounds may have occurred, causing low availability of the amino acid lysine for protein synthesis. This may cause an imbalance in the other available amino acids therefore the excess amino acids will undergo catabolism resulting in an increase in nitrogen excretion. The recommended lysine concentration used in this experiment was 50g/kg diet according to NRC, 1994. If this concentration was below requirement for the broiler chicks in this experiment then this may have also caused an amino acid imbalance.

It can therefore be concluded from this experiment that the objectives were not achieved due to poor digestibility and availability of the protein. Possible reasons for this are that the protein source of poultry offal may have

undergone intensive heat treatment causing low digestibility at high protein concentrations. This has therefore resulted in poor growth rates and poor nitrogen retention. However the study of digestibility and availability of protein offal is not the objective of this experiment. There is still the need to determine the response of broiler chicks to an increasing ideal protein and to determine an optimum lysine concentration.

CHAPTER 6

**THE EFFECT OF DIFFERENT PROTEIN CONCENTRATIONS
CONTAINING DIFFERENT LYSINE LEVELS ON NITROGEN
UTILISATION IN GROWING CHICKS.**

6.1 OBJECTIVES

The previous experiment explored the effect of an ideal balanced protein on the efficiency of nitrogen utilisation in the broiler chick. The major problem which was encountered, was the digestibility of the protein source used; poultry offal. As there are problems determining an ideal protein using practical broiler diets the objective of this experiment was to quantify the efficiency of nitrogen retention of broiler chickens given eight protein diets containing differing lysine concentrations. The protein in all the diets was approximately equal to the ideal essential amino acid concentration for this age of broiler chicken.

The aim of this experiment was to determine an optimum lysine concentration and optimum protein concentration and investigate the hypothesis that maximum weight gain and maximum efficiency of nitrogen utilisation occur at different lysine and protein concentrations.

6.2 MATERIALS AND METHODS

6.2.1 Treatments

Diets with eight crude protein concentrations of 129.0, 155.0, 173.0, 199.0, 217.0, 243.0, 278.0 and 305.0 g/kg diet were formulated (Table 6.1). The increasing protein concentration was achieved by the addition of maize gluten meal in replacement for maize. Six diets of each protein concentration were formulated to contain lysine concentrations of 40.0, 45.0, 50.0, 55.0, 60.0 or 65.0 g/kg CP by the addition of lysine hydrochloride. Diets were otherwise similar in metabolisable energy, calcium, phosphorus, linoleic acid and vitamin concentrations.

6.2.2 Bird Management

Two time replicates were performed due to not enough cages. Two hundred female Cobb hybrid broiler chickens were reared to 16 days old in a single group and housed in a light and temperature controlled environment. A proprietary diet and water was provided *ad libitum*. At the start of the experimental period 96 birds (for each time replicate) were weighed and placed in cages (two birds per cage). The cages (37 cm x 37 cm) were placed in a controlled environment room kept at 24°C and a relative humidity of

85% ($\pm 5\%$). 23 hours of light was given each day and food and water was provide *ad libitum*.

Table 6.1 Experimental diets

Ingredients	Crude Protein Concentrations (g/kg diet)							
	129	155	173	199	217	243	278	305
Maize Gluten	92	141	132	223	256	305	371	420
Maize	847	799	767	718	686	638	573	525
Limestone	19	20	20	20	21	21	22	22
Dicalcium Phosphate	13	13	12	12	12	13	10	10
Salt	4	4	4	4	4	4	4	4
Vitamin mineral Premix	9	9	9	9	9	9	9	9
Soya Oil	12.6	10.7	9.5	7.6	6.3	4.4	1.9	0
Lysine	2.6	3.5	4.1	4.9	5.5	6.4	7.5	8.4
Tryptophan	0.3	0.4	0.5	0.5	0.6	0.7	0.8	0.9
Threonine	0.2	0.3	0.3	0.4	0.5	0.5	0.6	0.7
<u>Calculated composition</u>								
ME (MJ/kg)	13.8	13.8	13.8	13.8	13.8	13.8	13.8	13.8
Crude Protein (g/kg diet)	129	155	173	199	217	243	278	305
Lysine (g/kg CP)	40	40	40	40	40	40	40	40
Methionine and Cystine	42	42	42	42	42	42	42	42
Threonine	35	35	35	35	35	35	35	35
Tryptophan	9	9	9	9	9	9	9	9

Vitamin and Mineral Premix (Ian Hollows Broiler No 1: Vitamin A 800000iu/kg, Vitamin D₃ 150000 iu/kg, Vitamin E 1250 iu/kg, Copper 800mg/kg, Selenium 10 mg/kg.

A 14 day feeding period was given. On day 0 and 14 the birds were weighed and feed intakes were measured for the last 3 days of the feeding period. Total excreta were collected on the last 4 days of the feeding period. The excreta were removed from every tray each 24 hour period and used for nitrogen determination as previously described.

6.2.3 Sample analysis

Analysis of feed and excreta samples for nitrogen content and faecal samples for uric acid content, took place using the procedures described in the previous chapter.

6.2.4 Statistical analysis

A randomised block analysis of variance was used. The two time blocks and also the four tier levels of the cages were used as blocking factors. The treatment means were compared in a factorial design; eight dietary protein concentrations and six dietary lysine and their interactions were compared. Differences between dietary protein and lysine concentrations were further compared by partitioning their sums of squares into linear and non-linear effects.

6.3 RESULTS

Weight gains (Table: 6.2) increased ($p<0.001$) with an increase in dietary protein concentrations, resulting in a curvilinear response (Figure: 6.1) . The protein concentration at which maximum weight gain occurred was calculated by differentiation of the quadratic equation to indicate an optimum of 280g/kg Crude Protein. Dietary protein concentration had a significant quadratic effect ($p<0.05$) on feed intake (Table: 6.3). Therefore an increase in protein concentration caused an increase ($p<0.001$) in feed conversion efficiency (FCE) resulting in a curvilinear response (Figure: 6.2).

Increasing the dietary protein concentration resulted in increased ($p<0.001$) total uric acid production (Table: 6.5), however increasing dietary protein concentration resulted in lower ($p<0.01$) protein digestibility (Table: 6.4) therefore an increase ($p<0.001$) in nitrogen excreted in the faeces (Table: 6.7). Protein concentration had no significant effect on the percentage digestible nitrogen retained (Table: 6.8).

Dietary lysine concentration had no significant effect on weight gain or feed intake in this experiment and a quadratic effect on FCE. However this was

mostly due to the bird responses when fed the dietary lysine concentration of 40g/kg CP. This data seemed anomalous and so a second analysis of variance was completed in which these data were removed. When removed, an increase in lysine concentration resulted in a significant ($p<0.01$) quadratic effect on weight gain (Figure: 6.3) and a significant ($p<0.05$) quadratic effect on feed conversion ratio (Figure: 6.4) indicating optimum lysine concentrations of 55g/kg CP.

Lysine concentration resulted in a significant quadratic effect ($p<0.01$) on uric acid production, but no significant effect on digestibility, digestible losses and the proportion of digestible nitrogen retained.

Table 6.2: Effect of protein and lysine concentrations on weight gain (g/day)

PROTEIN CONCENTRATION g/kg DIET		LYSINE CONCENTRATION g/kg CRUDE PROTEIN						
		40.00	45.00	50.00	55.00	60.00	65.00	Mean
129.00		31.43	24.76	40.10	22.47	25.75	31.22	29.29
156.00		36.11	36.73	26.73	37.55	38.35	27.42	33.82
173.00		36.67	40.84	37.04	35.90	44.07	43.17	39.61
189.00		30.59	31.57	45.61	43.52	37.52	35.90	37.45
215.00		45.71	38.54	45.03	39.39	37.86	34.43	40.16
241.00		46.48	34.65	39.02	50.67	52.45	44.09	44.56
275.00		50.66	44.23	48.78	50.01	46.88	44.54	47.52
300.00		39.57	49.64	43.25	47.83	38.58	36.44	42.55
Mean		39.65	37.62	40.63	40.92	40.18	36.20	

Lysine: SEM (141 r.d.f.) = 1.747 Protein: SEM (141 r.d.f.) = 2.018 Lysine-Protein: SEM (141 r.d.f.) = 4.942

Table 6.3: Effect of protein and lysine concentrations on feed intakes (grams per day).

		LYSINE CONCENTRATION g/kg CRUDE PROTEIN							
		40.00	45.00	50.00	55.00	60.00	65.00	Mean	
PROTEIN CONCENTRATION g/kg DIET	129.00	145.7	123.1	153.2	112.9	138.1	111.7	130.8	
	156.00	150.0	154.0	133.8	143.8	159.2	143.4	147.4	
	173.00	151.5	148.7	148.1	141.2	146.8	151.9	148.0	
	189.00	126.6	107.3	152.7	130.6	144.7	142.3	134.0	
	215.00	144.8	131.2	148.2	128.7	140.5	129.1	137.1	
	241.00	122.9	123.9	131.3	149.6	153.7	130.2	135.3	
	275.00	136.0	140.2	148.1	149.7	147.1	145.4	144.4	
	300.00	127.4	136.7	125.0	130.2	118.8	111.1	124.9	
	Mean	138.1	133.1	142.5	135.8	143.6	133.1		

Lysine: SEM (141 r.d.f.) = 3.960 Protein: SEM (141 r.d.f.) = 4.580 Lysine-Protein: SEM (141 r.d.f.) = 11.210

Tale 6.4: Effect of protein and lysine concentrations on feed conversion efficiency values.

	LYSINE CONCENTRATION g/kg CRUDE PROTEIN						
	40.00	45.00	50.00	55.00	60.00	65.00	Mean
PROTEIN CONCENTRATION g/kg DIET	129.00	0.2120	0.1954	0.2819	0.1919	0.1840	0.1801
	156.00	0.2399	0.2382	0.1956	0.2676	0.2399	0.1936
	173.00	0.2462	0.2753	0.2485	0.2583	0.2980	0.2879
	189.00	0.2371	0.2703	0.2996	0.3296	0.2596	0.2534
	215.00	0.3141	0.2883	0.3018	0.3107	0.2676	0.2664
	241.00	0.3831	0.3008	0.2988	0.3438	0.3374	0.3338
	275.00	0.3753	0.3177	0.3327	0.3370	0.3165	0.3087
	300.00	0.3138	0.3603	0.3567	0.3654	0.3335	0.3205
	Mean	0.2902	0.2808	0.2894	0.3005	0.2796	0.2681

Lysine: SEM (141 r.d.f.) = 0.01003 Protein: SEM (141 r.d.f.) = 0.01158 Lysine-Protein: SEM (141 r.d.f.) = 0.02837

Table 6.5: Effect of protein and lysine concentrations on total uric acid production (grams per day).

PROTEIN CONCENTRATION g/kg DIET	LYSINE CONCENTRATION g/kg CRUDE PROTEIN						
	40.00	45.00	50.00	55.00	60.00	65.00	Mean
129.00	1.299	1.059	0.527	0.589	0.786	0.605	0.811
156.00	0.457	1.017	1.198	1.142	1.682	1.095	1.098
173.00	1.455	1.259	1.402	2.253	1.145	0.881	1.399
189.00	1.112	1.926	1.931	1.475	1.667	1.170	1.547
215.00	1.663	1.380	1.589	2.064	1.264	1.470	1.572
241.00	1.634	1.533	1.767	1.464	1.918	1.258	1.596
275.00	1.902	1.287	2.182	2.112	1.821	1.802	1.851
300.00	2.296	1.591	2.163	2.167	1.873	2.235	2.054
Mean	1.477	1.381	1.595	1.658	1.519	1.314	

Lysine: SEM (141 r.d.f.) = 0.1189 Protein: SEM (141 r.d.f.) = 0.1373 Lysine-Protein: SEM (141 r.d.f.) = 0.3364

Table 6.6: Effect of protein and lysine concentrations on nitrogen excreted in the faeces (grams per day).

	PROTEIN CONCENTRATION g/kg DIET	LYSINE CONCENTRATION g/kg CRUDE PROTEIN							
		40.00	45.00	50.00	55.00	60.00	65.00	Mean	
	129.00	0.881	1.013	1.041	1.118	1.098	0.921	1.012	
	156.00	1.431	1.109	1.214	1.148	1.380	0.684	1.161	
	173.00	1.348	1.291	1.284	1.750	1.330	1.397	1.400	
	189.00	1.394	1.440	1.356	1.147	1.406	1.975	1.453	
	215.00	2.043	1.670	1.492	2.035	1.490	1.972	1.784	
	241.00	2.135	1.209	1.796	2.036	2.170	2.124	1.912	
	275.00	2.747	2.176	2.779	2.917	2.581	2.610	2.635	
	300.00	2.010	2.666	2.421	3.043	1.690	2.536	2.394	
	Mean	1.749	1.572	1.673	1.899	1.643	1.778		

Lysine: SEM (141 r.d.f.) = 0.1116 Protein: SEM (141 r.d.f.) = 0.1288 Lysine-Protein: SEM (141 r.d.f.) = 0.3156

Table 6.7: Effect of protein and lysine concentrations on protein digestibility (%).

	LYSINE CONCENTRATION g/kg CRUDE PROTEIN							
	40.00	45.00	50.00	55.00	60.00	65.00	Mean	
PROTEIN CONCENTRATION g/kg DIET	129.00	71.56	67.56	68.58	53.22	16.82	64.96	64.62
	156.00	58.76	70.16	63.81	67.81	65.07	80.79	67.73
	173.00	68.51	67.13	67.73	63.51	67.15	65.58	66.60
	189.00	63.21	60.96	70.15	70.46	69.00	56.81	65.10
	215.00	58.87	61.79	69.95	54.90	68.70	55.91	61.69
	241.00	56.92	76.06	62.62	66.60	61.68	58.87	63.79
	275.00	54.36	66.25	59.02	56.24	61.38	58.82	59.35
	300.00	66.50	60.43	60.48	50.70	69.25	53.08	60.07
	Mean	62.34	66.00	65.29	60.43	65.51	61.85	

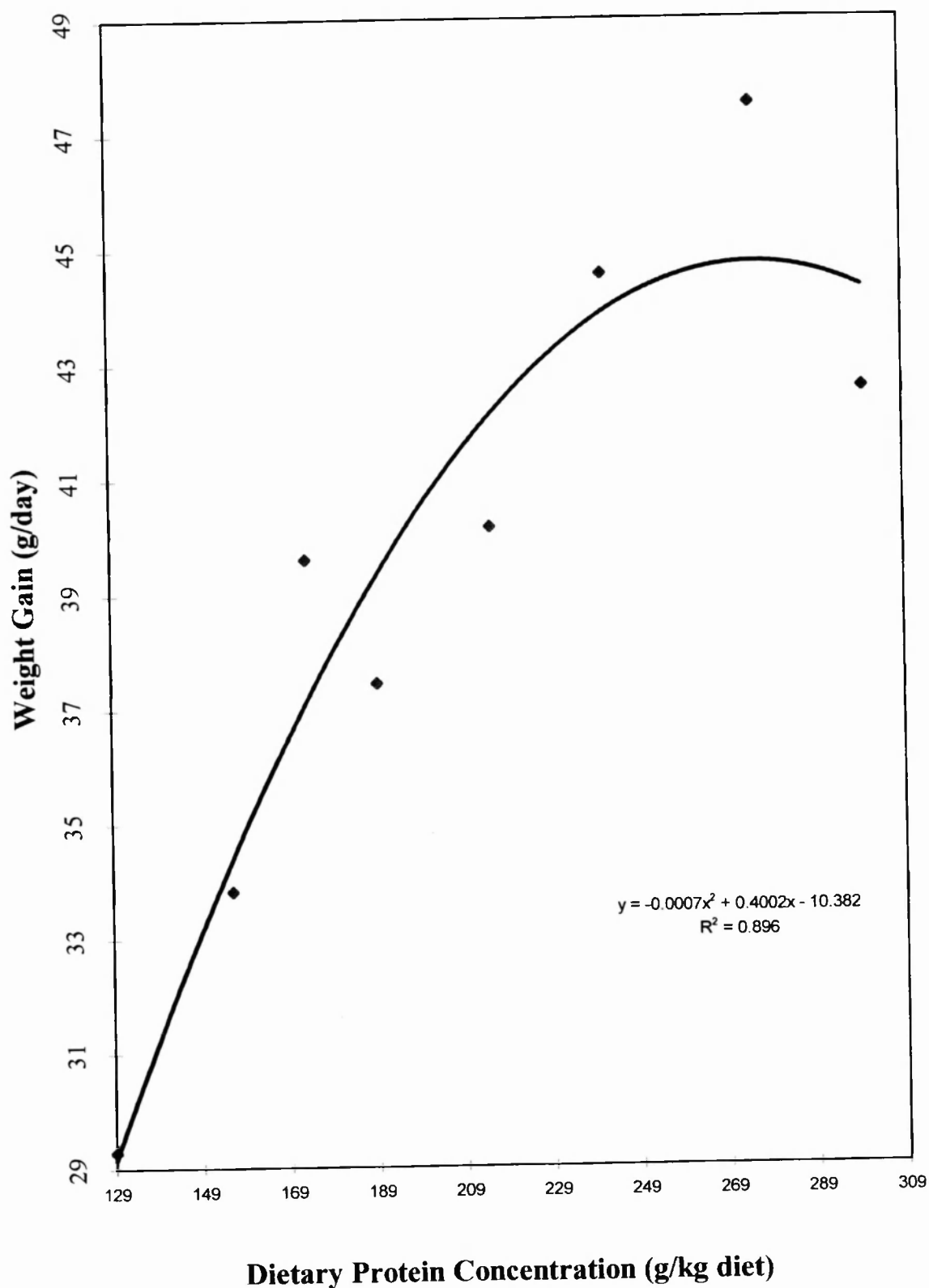
Lysine: SEM (141 r.d.f.) = 2.057 Protein: SEM (141 r.d.f.) = 2.375 Lysine-Protein: SEM (141 r.d.f.) = 5.817

Table 6.8: Effect of protein and lysine concentrations on the proportion of digestible nitrogen retained (%).

	LYSINE CONCENTRATION g/kg CRUDE PROTEIN							
PROTEIN CONCENTRATION g/kg DIET		40.00	45.00	50.00	55.00	60.00	65.00	Mean
	129.00	75.94	79.67	90.37	75.26	79.45	86.39	81.18
	156.00	90.32	83.95	77.06	80.63	71.88	84.08	81.32
	173.00	78.94	80.50	78.59	66.72	82.62	87.16	79.09
	189.00	81.66	57.65	75.63	77.36	78.80	81.26	75.39
	215.00	76.56	77.78	80.08	65.22	84.07	76.60	76.72
	241.00	75.81	83.08	74.97	84.25	77.94	82.70	79.79
	275.00	76.34	86.84	77.33	75.70	83.66	81.03	80.15
	300.00	76.63	85.42	75.83	72.61	79.10	67.30	76.15
	Mean	79.03	79.36	78.73	74.72	79.69	80.81	

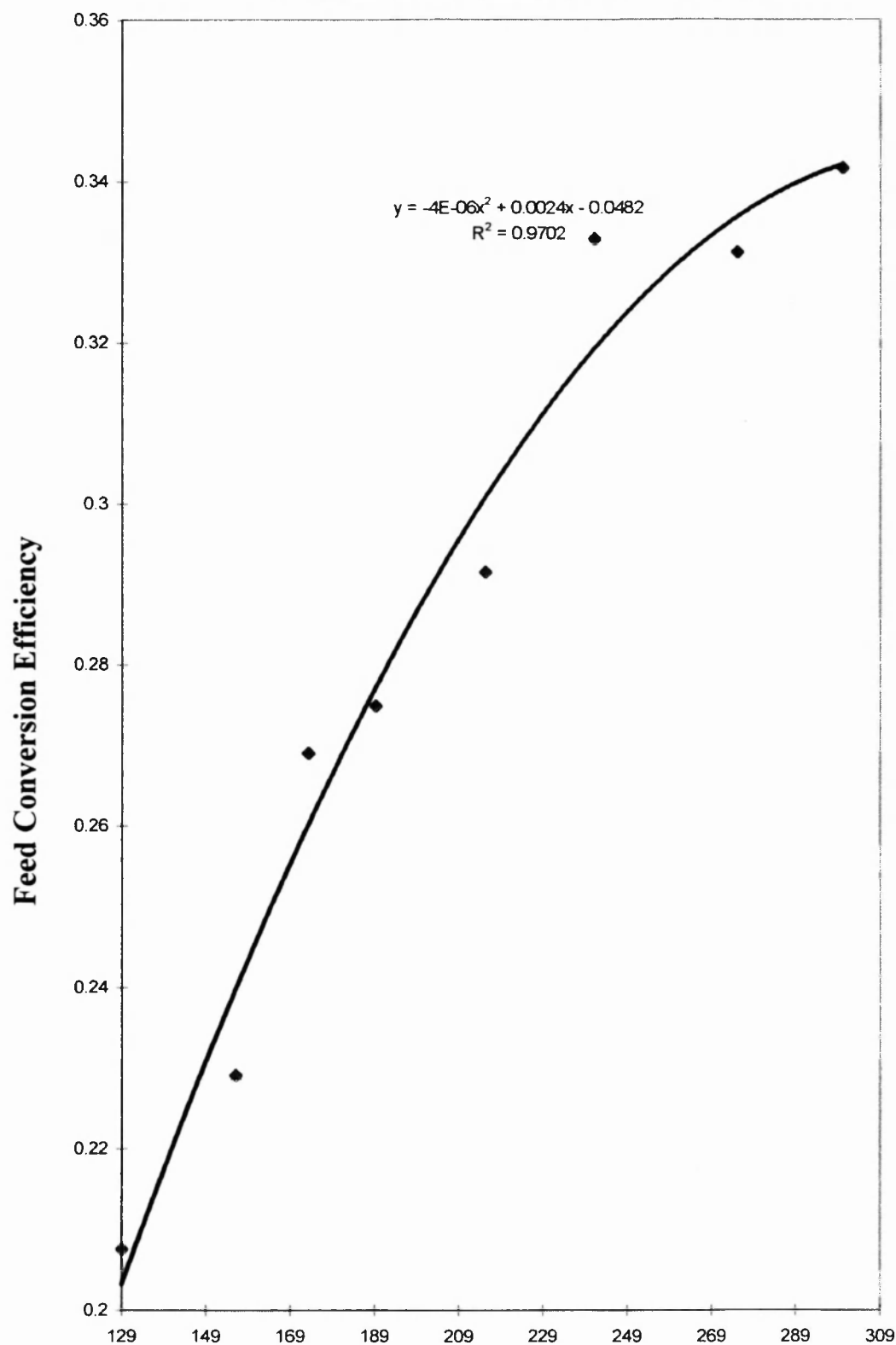
Lysine: SEM (141 r.d.f.) = 1.888 Protein: SEM (141 r.d.f.) = 2.180 Lysine-Protein: SEM (141 r.d.f.) = 5.341

Fig 6.1: Effect of dietary protein concentration
on weight gain (g/day)



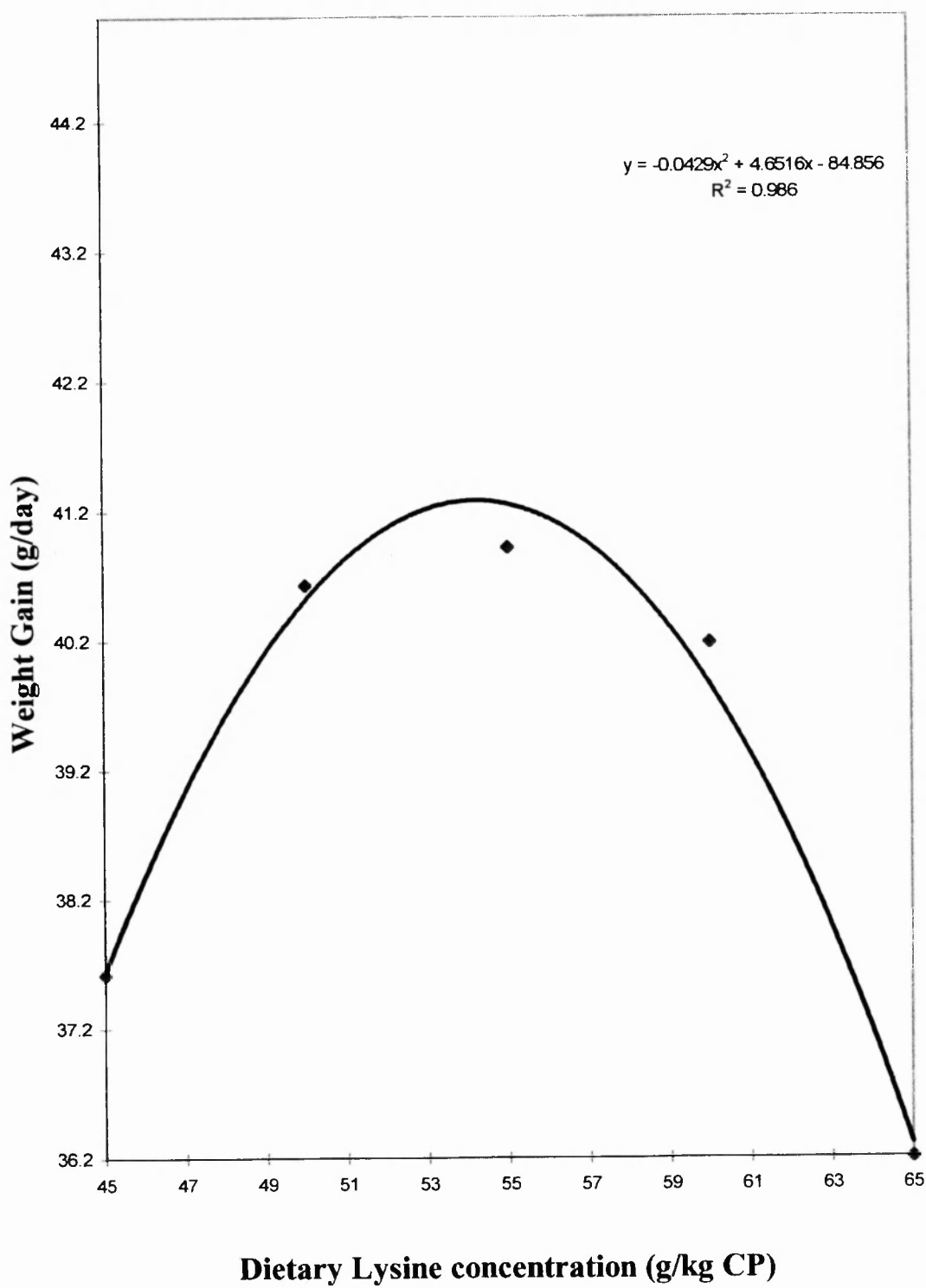
SEM (141 r.d.f.) = 2.018

Fig 6.2: Effect of dietary protein concentration on feed conversion efficiency



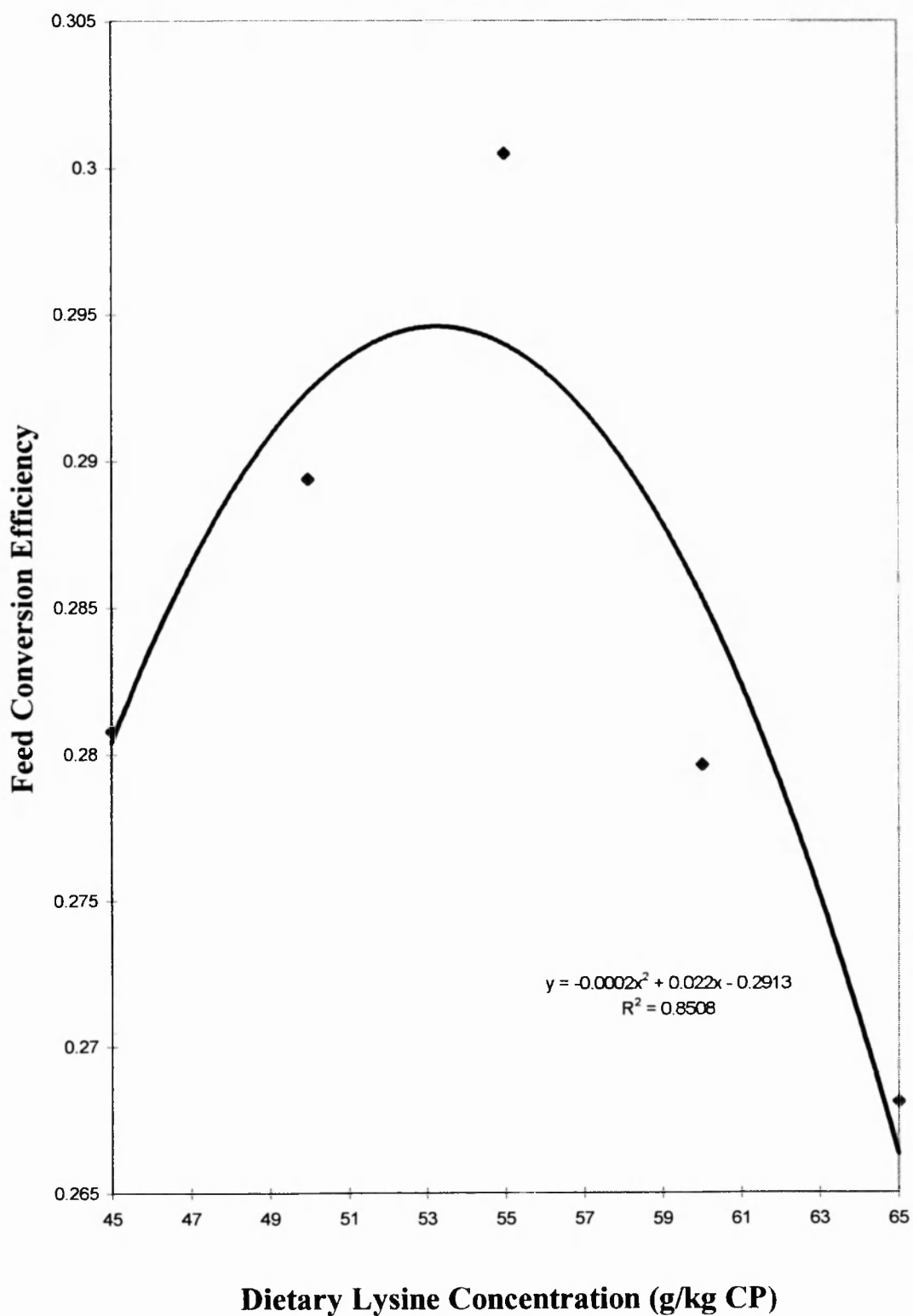
SEM (141 r.d.f.) = 0.1158 **Dietary Protein Concentration (g/kg diet)**

Fig 6.3 Effect of dietary lysine concentration on weight gain (g/day)



SEM (141 r.d.f.) = 1.747

Fig 6.4: Effect of dietary lysine concentration on feed conversion efficiency



SEM (141 r.d.f.) = 0.1003

6.4 DISCUSSION

Although there was an increase in weight gain with an increase in dietary protein concentration, these increases were poor compared with published data. The data was very variable, however it indicated a maximum weight gain at 275g/kg crude protein (Figure: 6.1), which is high according to published data; the recommended dietary protein concentration for broilers of 3-6 weeks of age is 200 g/kg diet (NRC 1994). However there is a linear decrease ($p < 0.001$) in total protein digestibility with increasing dietary protein concentration. This indicates that less of the protein was available to the chick. This could therefore explain the reason why such a high optimum amount of dietary protein was observed. The increase in protein concentration caused an increase ($p < 0.001$) in feed conversion efficiency resulting in a curvilinear response. Due to variability it was difficult to determine an optimum response, however the results indicated that the optimum occurred at 241 g/kg CP (Figure: 6.2).

The maximum weight gain and maximum FCE occurred at the dietary lysine concentration of 55g/kg CP (Of the range of 45 to 65 g/kg CP). This is higher than the lysine concentration recommended by NRC 1994 (50g/kg CP)

but is in close agreement with the optimum found by Morris *et al.* (1987). A second estimate from Morris and Abebe (1990) was 56g/kg CP for maximum growth and 57 g/kg CP for food efficiency.

It may therefore be concluded from this experiment that increasing the dietary protein concentration indicated a maximum weight gain at 275 g/kg diet, however due to no response of nitrogen utilisation, the hypothesis that maximum weight gain and maximum nitrogen retention occur at different protein concentrations has not been proved. Increasing the dietary lysine concentration resulted in an optimum weight gain at 55 g/kg CP however no response was observed with nitrogen utilisation therefore the hypothesis could not be proved.

Although optimum dietary protein concentration has been established and there was no interactions between protein and lysine concentrations, there is a further need to investigate optimum lysine concentrations using a wider variety of concentrations and the effects they have on nitrogen utilisation in the broiler chick. An investigation into the catabolism of lysine will determine the biological mechanisms behind lysine utilisation and further investigate optimum amino acid requirement.

CHAPTER 7

THE EFFECT OF DIFFERENT LYSINE CONCENTRATIONS ON NITROGEN UTILISATION IN GROWING CHICKS

7.1 OBJECTIVES

The previous experiment explored different protein and lysine concentrations to determine their optimal levels for weight gain, feed conversion efficiency and nitrogen utilisation. The objective of this experiment was to further explore a wide range of lysine concentrations to quantify the effect on maximum weight gain and maximum efficiency of nitrogen utilisation. The objective was to test the hypothesis that maximum weight gain and maximum nitrogen retention occur at different lysine concentrations.

The activity of the lysine catabolic enzyme lysine α -ketoglutarate reductase (LKGR) was also assessed to determine at what lysine concentration lysine catabolism occurs hence determine the lysine requirement. This method was modified and had not been used previously with the chick.

7.2 MATERIALS AND METHODS

7.2.1 Treatments

Eight dietary treatments of 200 g/kg dietary crude protein were formulated that contained lysine concentrations of either 30.0, 40.0, 50.0, 57.5, 65.0, 72.5, 80.0 or 100.0 g/kg CP (Table 7.1). Diets were otherwise similar in metabolisable energy, calcium, phosphorus, linoleic acid and vitamin concentrations.

7.2.2 Bird Management

One hundred female Cobb hybrid broiler chickens were group housed in a light and temperature controlled environment until 21 days of age. A proprietary diet and water was provided *ad. libitum*. At the start of the experimental period 96 birds were weighed and placed in cages (two birds per cage). The cages (37 cm x 37cm) were in a controlled environment room with light (23 hours per day), temperature (24° C) and a relative humidity of 85%(±5%).

A 14 day feeding period was given. On days 0 and 14 birds were weighed and feed intakes were measured from day 9 to day 14.

Total excreta were collected on the last 4 days in the same manner as described previously.

On day 14 of the feeding period, the birds were sacrificed by cervical dislocation. Fresh livers were excised using a scalpel followed by weighing, then rapidly freezing in liquid nitrogen. Each liver was stored at -20°C until required for analysis.

Table 7.1 Experimental diets

Ingredients	Lysine Composition (g/kg CP)							
	575	575	575	575	575	575	575	574.8
Wheat	575	575	575	575	575	575	575	574.8
Maize Gluten	165	165	165	165	165	165	165	165
Maize Germ Meal	132	132	132	132	132	132	132	132
Rape Meal	12	12	12	12	12	12	12	12
Full Fat Soya	57	57	57	57	57	57	57	57
Limestone	12	12	12	12	12	12	12	11.9
Dicalcium Phosphate	15	15	15	15	15	15	15	15
Salt	3	3	3	3	3	3	3	3
Vitamin Mineral Premix	20	20	20	20	20	20	20	20
Soya Oil	8	8	8	8	8	8	8	8
<u>Calculated composition</u>								
ME (MJ/kg)	13.14	13.14	13.14	13.14	13.14	13.14	13.14	13.14
Crude Protein	202	202	202	202	202	202	202	202
Lysine (g/kg CP)	30	40	50	57.5	65	72.5	80	100
Methionine and Cystine	37	37	37	37	37	37	37	37
Threonine	31	31	31	31	31	31	31	31
Tryptophan	8	8	8	8	8	8	8	8

7.2.4 Sample analysis

7.2.4.1 Nitrogen Analysis

The analysis of nitrogen content of feedstuffs and excreta samples was performed as described previously..

7.2.4.2 Uric acid analysis

The modified method of Pudalkiewicz *et al* (1967), was used in Section: 5.2.4.

7.2.4.3 Lysine Catabolic Enzymes

The development of this method involved a considerable amount of original work, so therefore the methods are described in detail. The activity of the lysine catabolic enzyme lysine α -ketoglutarate reductase (LKGR) was assayed using a modification of the method used by Foster *et al.* (1993).

7.2.4.3.1 Reagent Preparation

Homogenisation Buffer: (220mM mannitol, 70 mM sucrose, 2mM HEPES, 1mM EGTA, pH 7.8). 40.077 g of mannitol, 23.961g of sucrose, 0.4766g of HEPES and 0.3804g of EGTA were weighed and added to a litre volumetric

flask. The pH was corrected to 7.8 and made up to volume with deionised water.

Reaction mixture 1: (16.66mM α -ketoglutarate, 275.56 μ M NADPH in 0.1M HEPES containing 0.2% Nonidet P-40). 0.28005g of α -ketoglutarate, 0.02296g of NADPH, 2.383 g of HEPES and 0.2ml of nonidet P-40 were weighed into a 100ml volumetric flask, the pH corrected using NaOH and made up to volume using deionised water.

Reaction mixture 2: (0.88g of mitochondrial protein in 0.1M HEPES containing 0.2% Nonidet P-40). See below.

Reaction mixture 3: (36.5mg/ml Lysine in 0.1M HEPES containing 0.2% Nonidet P-40.): 0.9125g of Lysine, 0.5958g of HEPES and 0.05ml of Nonidet P-40 were weighed in a 25ml volumetric flask and made up to volume using deionised water.

7.2.4.3.3 Mitochondrial Preparation

The mitochondria were isolated according to Johnson and Lardy (1948).

Approximately 3 grams of frozen liver was homogenised in 27 ml of ice cold

homogenisation buffer (220mM mannitol, 70mM sucrose, 2mM HEPES and 1mM EGTA, pH 7.8) using a Potter Elvehjem hand held homogeniser. Homogenisation took place until liver tissue was no longer discernable and care was taken not to provide a vacuum so as to damage mitochondria.

The liver homogenate was differentially centrifuged to isolate mitochondria by modifying the method according to Johnson and Lardy 1967.

The liver homogenate was centrifuged at 600g for 10 minutes. The resulting supernatant was retained and 15 ml of 0.25M sucrose solution was added to the pellet, followed by centrifugation at 600g for 10 minutes. The washing with sucrose solution contributes to the yield and integrity of the final mitochondrial preparation by permitting the recovery of the larger mitochondria (Johnson and Lardy, 1967). The resulting supernatants were combined and centrifuged at 15,000 g for 5 minutes. The resulting supernatant was decanted along with lightly packed pink chromosomes and the pellet was washed with 15 ml 0.25M sucrose solution followed by centrifugation at 15000g for 5 minutes. This procedure took place a further two times with the resulting pellet being stored on ice.

5ml of 0.1M Hepes containing 0.2% Nonidet P-40, pH 7.8 was added to each pellet for resuspension purposes. The suspension was then analysed for protein content on a Technicon RA-1000 Blood Analyser (Bayer Diagnostics, Basingstoke, Hampshire).

The mitochondrial suspension was diluted with 0.1M Hepes containing 0.2% Nonidet P-40, pH 7.8 so as to provide a final concentration of 0.88 mg of mitochondrial protein per ml. This resulted in the formation of Reaction mixture 2.

7.2.4.3.4 Enzyme assay

The enzyme activities were measured by initial rate measurements of absorbance change against time at 340nm in a Beckman Spectrophotometer. The temperature of the cuvette chamber was maintained at 39°C using a circulating water bath.

450ml of Reaction mixture 1 (α -ketoglutarate and NADPH) and 450 μ l of reaction mixture 2 (mitochondrial fraction) were mixed in a cuvette and 10 minutes was allowed for the transfer of heat through the cuvette wall. The reaction was initiated by the addition of 100 μ l of Reaction mixture 3 (25mM

lysine). The decrease in absorbance at 340nm was read against a blank in which the volume of lysine was replaced by the equivalent volume of assay buffer (0.1M HEPES pH 7.8). The Spectrophotometer was blanked using deionised water.

The final assay volume was 1000 μ l and the molar extinction coefficient used for lysine ketoglutarate reductase was 6220. An absorbance change of 6.220 indicates the production of 1 μ mole of Saccharopine.

Enzyme activities were expressed as nmol/min.mg mitochondrial protein. by using the following equation.

7.2.5 Statistical Analysis

Randomised block analysis of variance was performed. The treatment (level of dietary lysine concentration) sum of squares were partitioned into their linear and non linear effects, using the Genstat computer package (Lawes Agricultural Trust 1990)

lysine). The decrease in absorbance at 340nm was read against a blank in which the volume of lysine was replaced by the equivalent volume of assay buffer (0.1M HEPES pH 7.8). The Spectrophotometer was blanked using deionised water.

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7.3 RESULTS

Lysine concentration had a significant curvilinear effect ($p < 0.01$) on weight gain in this experiment (Table: 7.2). The lysine concentration at which maximum weight gain occurred was obtained by regression analysis of a quadratic equation and was indicated at 64g/kg CP (Figure:7.1). Lysine concentration had no significant effect on feed intake.

Increasing dietary lysine content of the diet resulted in a significant ($p < 0.001$) quadratic effect on the proportion of digestible nitrogen being retained. The maximum proportion of nitrogen retained was indicated at 64g/kg CP (Figure 7.2).

The activity of the lysine catabolic enzyme LKGR was found to be linear but when a curvilinear equation was applied to the data, there was a significant increase in the proportion of variance resulting in a polynomial relationship (Figure 7.3). This relationship was consistent with the physiological response of published data which relates amino acid oxidation to the level of amino acid supply.

Table 7.2 : Effect of lysine content of the diet on weight gain, feed intake and feed conversion efficiency.

	Dietary Lysine concentration (g/kg CP)								
	30.0	40.0	50.0	57.5	65.0	72.5	80.0	100.0	SEM (35 r.d.f.)
Weight gain (g)	75.6	79.5	81.3	85.7	79.9	86.8	83.6	72.5	4.29
Feed intake (g)	274.0	295.4	262.7	291.3	260.2	290.0	274.0	245.1	13.69
Food Conversion Efficiency	0.2757	0.2707	0.3116	0.2933	0.3117	0.3003	0.3071	0.3008	0.0165

Table 7.3: The effect of lysine content of the diet on uric acid production, nitrogen excreted in the faeces, protein digestibility and nitrogen retained as a proportion of digestible nitrogen.

	Dietary Lysine Concentration (g/kg CP)									
	30.0	40.0	50.0	57.5	65.0	72.5	80.0	100.0	SEM (35 r.d.f.)	
Total uric acid excreted (g/day)	5.81	4.44	4.19	3.64	3.36	4.42	4.43	5.13	0.438	
Total nitrogen in faeces (g/day)	2.87	2.74	2.59	3.49	2.98	2.76	2.98	2.49	0.338	
Protein digestibility (%)	71.38	75.26	74.03	66.08	68.70	74.29	70.88	72.70	2.589	
Digestible nitrogen retention (%)	66.92	78.39	76.37	76.98	78.23	77.40	75.11	69.69	2.524	
Lysine oxidation (nmol/min/ μ g protein)	5.52	5.97	6.37	10.03	10.80	12.23	15.86	16.30	0.853	

Fig 7.1: Effect of dietary lysine content on weight gain (g/day)

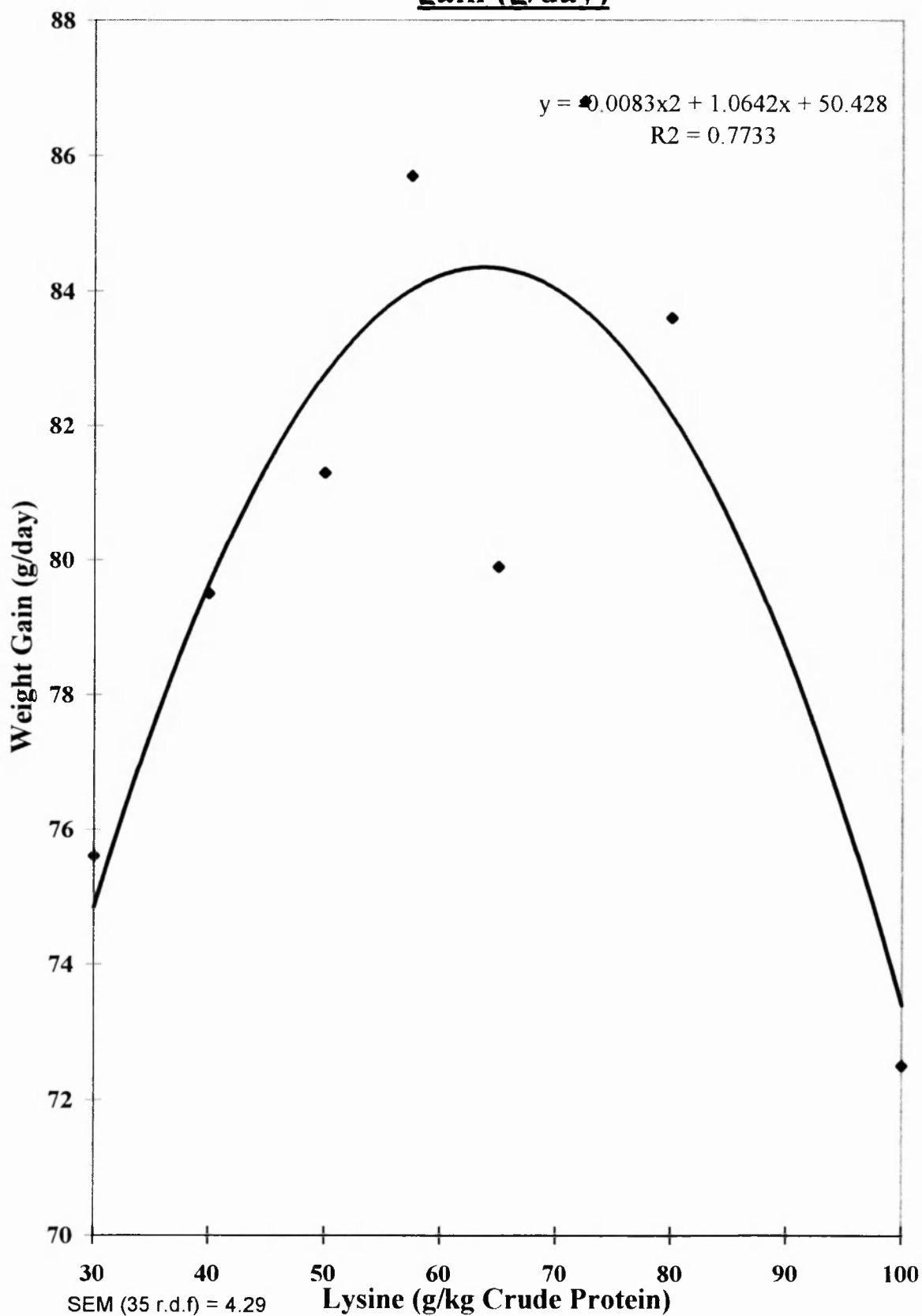


Fig 7.2: Effect of dietary lysine concentration on percent digestible nitrogen retention

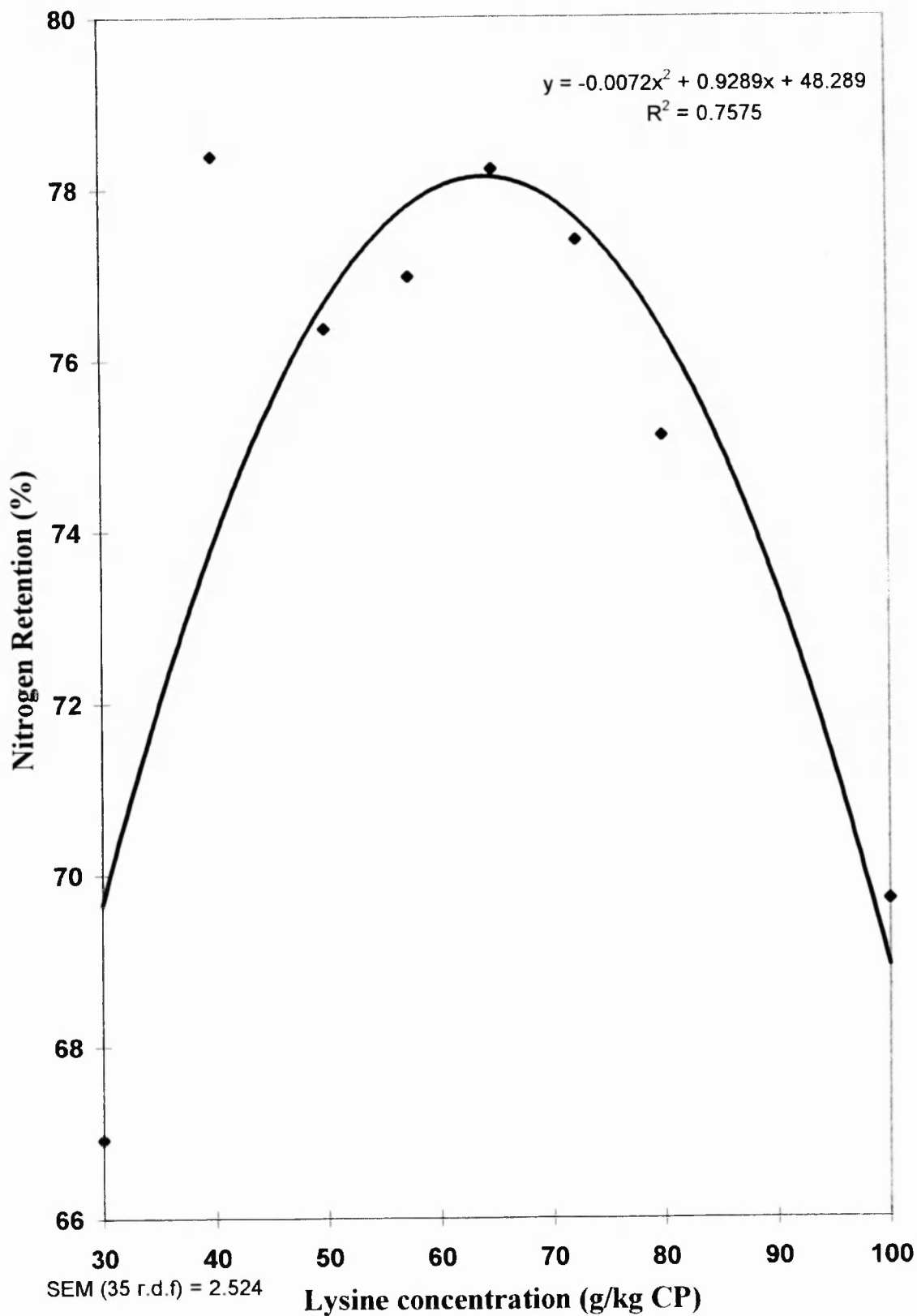
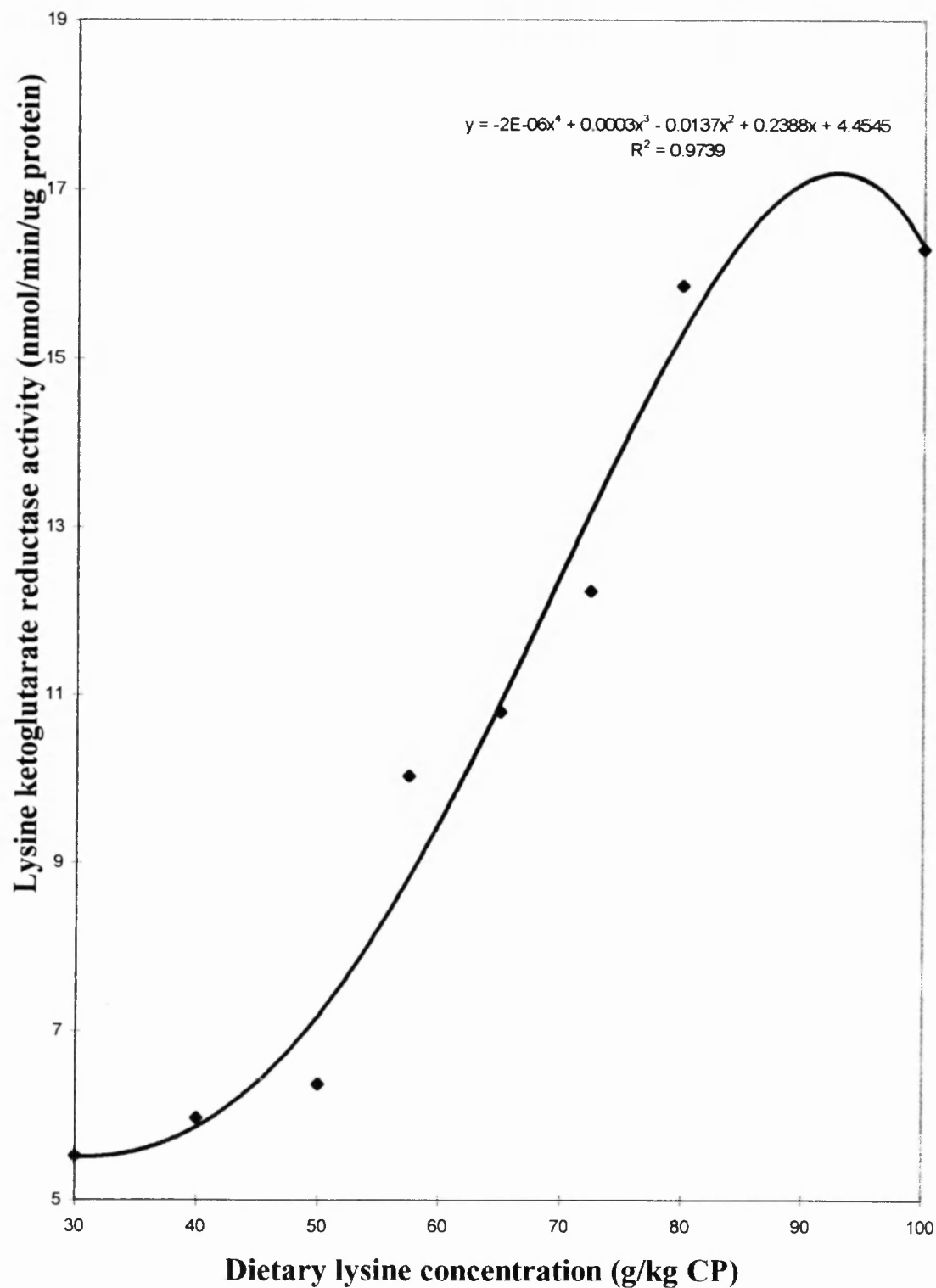


Figure 7.3: Effect of dietary lysine concentration on lysine ketoglutarate reductase activity



SEM (35 r.d.f) = 0.853

7.4 DISCUSSION

Due to the importance of this final experiment, this discussion will include previous experimental work and will be combined into a general discussion.

The results from this experiment are consistent with previously published work (Morris *et al.*, 1987; Morris and Abebe, 1990; Abebe and Morris, 1990b). However the calculated optimum values in the present experiment are not consistent with the conclusions of those authors.

As lysine concentration increases in this experiment there was an increase ($p < 0.001$) in weight gain to maximum that occurred at 64g/kg CP. This appeared higher than the optimum calculated in the previous experiment (55g/kg CP) and that of Morris *et al.*, (1987) who showed that the amount of lysine needed to maximise growth rate was a fixed proportion of the protein at 54 g/kg CP. Results from Morris and Abebe (1990b) show that lysine should be 56g/kg of the CP for maximum growth and 57g/kg for maximum efficiency.

However, Morris *et al.*, 1987 used synthetic and semi synthetic ingredients

in the basal diet, which would probably cause high availability of the lysine to the chick. High availability would therefore result in a lower requirement of the chick hence the lower optimum lysine concentration. The diet used in this study consisted of more practical ingredients which may have resulted in low availability of lysine hence the higher estimate of the ideal lysine concentration.

The method used to determine the optimum lysine concentration by Morris *et al.*, 1987 was to fit a quadratic equation to data relating growth rate to dietary lysine content at different protein concentrations. The maximum point on the parabola gave an optimum lysine concentrations which according to the authors may have been markedly influenced by the range of treatments chosen for the experiment (Morris *et al.*, 1983).

The range of lysine concentrations that Morris *et al.*, 1987 used was from 40g/kg to 60g/kg CP, as in the previous experiment. In this experiment, it may be possible to calculate a similar optimum if only 40 g/kg to 60 g/kg CP lysine concentrations were taken into account as was found in the previous experiment. However, when using a much wider range of lysine concentrations as in this experiment, the optimum level is clearly higher.

This experiment indicates a highly significant increase ($p < 0.001$) in percentage of digestible nitrogen retained with an increase in lysine concentration. The maximum level of nitrogen retained occurred at the lysine concentration of 64g/kg CP. Therefore there is no evidence that the maximum weight gain and maximum nitrogen retention occurred at different lysine concentrations.

This experiment utilised an enzyme assay to determine the activity of LKGR at the different dietary levels of lysine concentration. The concept of using an indicator amino acid to determine amino acid requirements was suggested by Kim *et al.*, (1983) using young pigs.

The present experiment indicated that as dietary lysine concentration increased, there was a significant linear increase ($p < 0.001$) in amino acid oxidation. A polynomial regression analysis gave a more accurate representation of the physiological response to amino acid supply. This equation indicated a peak lysine oxidation at approximately 50 g/kg CP (Figure:7.3) which is in agreement with the concentration recommended by NRC 1994 (50 g/kg).

Although the data was variable, this physiological response may be explained by previous published work regarding amino acid supply and amino acid oxidation in the rat. When the amino acid intake is below requirement it is used with relatively constant high efficiency (Beckett *et al.*, 1992). This also indicates that the lysine was being preferentially used for protein synthesis. When the amino acid supply is in excess of the requirement the increase in amino acid oxidation tends to be proportional to the level of the amino acids in the diet (Gahl *et al.*, 1991), hence the linear increase in lysine oxidation. This is consistent with research by Soliman and Harper, 1971; Brookes *et al.*, 1972; Kang-Lee and Harper, 1978, 1979 and Kim *et al.*, 1983.

However, the lysine requirement calculated using the enzyme assay is not consistent with the requirement for maximum growth or maximum nitrogen utilisation. These results indicate that the lysine is becoming oxidised before maximum protein deposition has occurred at the lysine concentration 64g/kg CP. Therefore protein synthesis may be exerting some sort of effect on lysine oxidation or vice versa.

A number of researchers have proposed a link between protein synthesis and amino acid oxidation. Millward and Rivers (1988), developed the anabolic

drive hypothesis which suggested that an over supply of amino acids and an increase in amino acid oxidation was required to obtain maximal rates of protein deposition. This also resulted in the statement that dietary indispensable amino acids serve an important transient function prior to their oxidation exerting a regulatory influence on maintenance and growth. This is consistent with this experimental data and may explain the lower amino acid requirement of 50 g/kg according to the enzyme oxidation assay. It is perhaps at this point where the lysine is beginning to undergo the process of amino acid oxidation therefore exerting its regulatory influence that causes the associated increase in protein synthesis to the optimum weight gain at 65 g/kg CP.

Another theory is that an increase in amino acid oxidation is due to the increase in protein synthesis which could be the result of increased cycling of protein (Hawkey, 1994). Due to amino acids being constantly recycled, amino acids resulting from protein breakdown may enter the free pool or be directly used for protein synthesis (Barnes *et al.*, 1992) whilst amino acids in the free pool may be removed for incorporation into protein. This theory is also consistent with the present experimental data. Increased protein supply stimulates protein synthesis and degradation (Waterlow *et al.*, 1978) which

would therefore increase the rate of protein turnover.

The proposed link between protein synthesis and amino acid oxidation may also be due to the synthesis of amino acid oxidation enzymes resulting in an increase in the oxidative capacity of the system. Hawkey (1994) speculated that the transcription of the genes responsible for increased protein synthesis are linked to the expression of the catabolic enzymes for amino acids. This prior induction of amino acid oxidative enzymes by rats fed a high protein diet has been observed for tyrosine threonine and histidine (Ip and Harper 1973; Kang-Lee and Harper 1978, 1979).

It is the above theories of linkage between amino acid oxidation and protein synthesis in relation to amino acid supply which may explain the relationship observed in this experiment.

CHAPTER 8

CONCLUSIONS

i) The first two experiments gave broiler growth responses, due to increases in protein concentration and lysine concentration, that were consistent with previously published literature. Although these experiments examined a large number and range of dietary concentrations, the large variability of the data made it difficult to accurately describe the response curves.

ii) The optimum crude protein concentration for maximum growth was indicated at 275 g/kg diet and there was no effect of protein concentration on the efficiency of nitrogen utilisation. The optimum lysine concentration for maximum growth was indicated to be 55g/kg CP.

iii) The third experiment indicated that the optimum lysine concentration

was 64g/kg CP and the optimum for maximum efficiency of nitrogen utilisation was also indicated at 64 g/kg CP. Therefore there was no evidence that maximum weight gain and maximum nitrogen retention occurred at different protein and lysine concentrations.

iv) Lysine oxidation increased with an increase in dietary lysine and there was evidence of a curvilinear response. A rapid increase in lysine oxidation was indicated at 55g/kg CP. This data therefore gives some support to the theories of poor relationships between the rates of protein synthesis and amino acid oxidation although the variability of the data did not allow for a precise estimate of point of increase of amino acid oxidation.

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